STRUCTURAL CHEMISTRY OF ALGAL POLYSACCHARIDES

by

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ABSTRACT OF THESIS

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Title of Thesis Structural Chemistry of Algal Polysaccharides.

Section 1. Studies on the Polysaccharides of Ulva lactuca.

1. Cold and hot water extractions of the green seaweed Ulva lactuca afforded a sulphated polysaccharide extract (ca 20 % of dry weight of weed), $[\alpha]_D$-49°, sulphate,16%, uronic anhydride,15.5%) composed of glucose xylose, rhamnose, mannose(trace) and glucuronic acid.
2. A pure starch-type glucan(ca 1% yield) was separated from this extract by iodine precipitation. The residual polysaccharide($[\alpha]_D$-70.7°, sulphate 19%, uronic anhydride,17.6%) consisted of (molar proportions) glucose (1.0),xylose(3.4),rhamnose(6.9) and trace of mannose.
3. The sulphated polysaccharide was subjected to a variety of fractionation procedures which indicated the apparent essential homogeneity of the material. With DEAE-cellulose, however, a molecular fractionation was obtained.
4. The polysaccharide was desulphated with dry methanolic hydrogen chloride(ca.0.1N) to give a product($[\alpha]_D$-80°,sulphate 0.3%,uronic anhydride 20.5%) consisting of (molar proportions) glucose(1.0),xylose(2.3), and rhamnose(3.2).
5. The low reduction of periodate of the sulphated and desulphated polysaccharide indicated a high proportion of 1,3-linked residues. The higher reduction by the latter revealed the presence of a larger number of free glycol groups in the desulphated material. Determination of the molar proportions of the unattacked sugars in the respective oxopolysaccharides indicated that a higher proportion of rhamnose units was attacked in the desulphated polysaccharide. These results together with those of infrared analysis provide evidence that a high proportion of the sulphate groups in the polymer are located at C_2 in rhamnose.
6. A small proportion of D-arabinose and a trace of lyxose(probably D-lyxose) were separated after alkali treatment of the sulphated polysaccharide and subsequent acid hydrolysis. By the action of sodium methoxide on the alkali treated polysaccharide followed by acid hydrolysis, a small amount of 2-O-methylxylose was formed. This,together with the formation of arabinose on treatment with alkali, is proof that some of the xylose units in the polymer are sulphated at position 2. The trace of lyxose probably arose from epoxide ring migration on xylose end groups(initially sulphated at C_2) during the alkali treatment of the polysaccharide.

Section 2. The Constitution of Alginic Acid.

1. Mannuronic and glucuronic acid were shown to undergo epimerisation at C_5 under alkaline , but not under neutral conditions.
2. Two chemically similar fractions were obtained on elution of alginic acid on a column of DEAE-cellulose.
3. Preparation of the 2,3-di-n-propionate of alginic acid(extracted from Laminaria digitata) and subsequent diborane reduction produced a polysaccharide($[\alpha]_D$-87.5°,uronic anhydride 9.6%).
4. From a partial acid hydrolysate of this reduced material a pure disaccharide was separated. This was partially characterised as a mannosylgulose.

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I N T R O D U C T I O N

G E N E R A L

With very few exceptions all the plants which grow in the sea belong to the algae. Among the members of this group the seaweeds will be the most familiar to the ordinary observer. The algae are recognised as one of the most primitive groups within the plant kingdom. They evolved early in the botanical history of the earth, and their present-day forms do not seem to differ much from their ancestral ones. Thus the seaweeds, like the rest of the algae, show only a small degree of differentiation, and contrary to the higher plants they do not possess true roots, stems or leaves. This, however, does not prevent considerable differences between the various groups, e.g. in shape, colour, size etc. Some seaweeds rank among the most gigantic members of the lower plants, while others are almost invisible as individuals to the naked eye.

The classification of the algae conveniently follows the colour, as differences in the pigments generally coincide with important morphological distinctions. Thus the algae are divided into four main groups: The brown (Phaeophyceae), red (Rhodophyceae), green (Chlorophyceae) and blue-green (Cyanophyceae) algae. Like the land plants they all possess chlorophyll and require light and carbon dioxide to perform photosynthetic activity.

The seaweeds are widely distributed along the coastal areas

of the world, occurring in immense quantities. As these plants generally contain protein, mineral salts and vitamins in addition to carbohydrates, they should be of significant nutritional importance. And in fact the use of certain seaweeds for human and animal consumption has gone on for many hundreds of years. This is especially true in the case of China, Japan and other countries in the far East. In Europe seaweeds as such are no longer commonly used as human food, but have found some utilization in feedstuffs for domestic animals.

In previous times algae were harvested in large amounts for the kelp industry (the production of iodine, potash and soda). This is now non-existant since these materials can be obtained more cheaply and more readily from other sources. The industrial interest in seaweeds today is mainly based on the polysaccharide content and the ability of these polymers to form gels or viscous solutions with water. From this viewpoint members of the Rhodophyceae and Phaeophyceae are the most important, partly because of the particular materials they contain, and partly because they occur in sufficient quantities to possess an economic value. As a consequence of this the polysaccharides of the brown and red seaweeds have been more thoroughly investigated than those of the green and blue-green algae.

ALGAL POLYSACCHARIDES (1) (2) (3).

The polysaccharides of the plants living in the sea are in some respects different from the polysaccharides of the land

plants. This is not surprising when one considers the completely different conditions under which these plants live and the distant botanical relationship between them. In the land plants the cell walls are normally composed of cellulose, hemicellulose and lignin - materials which render the rigidity characteristic of the normal plant cell. Seaweeds however require skeletal units which will give flexibility and ease of movement. Therefore there is a high proportion of mucilaginous materials in the cell wall, and cellulose and lignin are either absent or present in very small quantities.

The polysaccharides of the marine algae may be conveniently divided into food reserve materials and polysaccharides of structural significance. The first type is exemplified by the β-1,3-linked glucan, laminarin, in the brown algae and by glucose polymers resembling starch in the green and red algae. The proportions present of these reserve carbohydrates differ - as one might expect - considerably with the seasons. The structural polysaccharides are more complex and frequently contain a number of different sugars. A characteristic feature of these polymers is their acidic nature. This acidity arises from the presence of uronic acid residues and/or sulphuric acid half ester groups. Sulphate ester groups have so far not been found in the carbohydrates of land plants, but they are present in some of the animal polysaccharides. Thus the chondroitin sulphates and the natural blood anticoagulant, heparin, are sulphated. The presence of sulphate groups in the seaweeds may

serve different purposes. It is possible that as a result of their strongly hydrophilic nature they promote retention and absorption of water and so prevent excessive dehydration of these plants on their exposure at low tide. In support of this theory it was found that a sample of the green seaweed Ulva lactuca which had grown in deep water and thus had never been exposed to open air, had a lower sulphate content than a sample collected in the tide belt.

A complicating factor in the structural work on algal polysaccharides is that these polymers do not seem to have a constant molecular structure. It appears that the algae can modify to a minor extent the composition and structure of their mucilaginous polysaccharides in such a way as to make them more suitable for particular environmental conditions. Thus the composition of the polysaccharide material from the same botanical species will probably vary with the seasons of the year, with the location in which the alga is growing, with the composition of the seawater etc. This fact must be considered when comparisons are made of polysaccharide materials isolated from botanically identical or related species. But while these dissimilarities may cause major quantitative differences in the polysaccharide constituents, it is thought that they only result in minor modifications in molecular structure and the overall constitution of polysaccharides synthesised by a single species always appears to be the same.

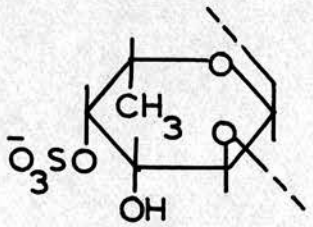
Too little is known about the nature of the polysaccharides

of blue-green algae to state whether they are similar to those of the other groups. It is notable in the other three main groups that the botanical classification into brown, red and green algae also corresponds to a chemical classification, each group synthesising its own characteristic polysaccharides. A brief review of the present state of knowledge concerning the constitution of the more important polysaccharides in those three groups will serve to illustrate their essential dissimilarity.

Phaeophyceae.

The algae belonging to this group contain alginic acid, fucoidin and laminarin as their major polysaccharides. Alginic acid forms the subject matter of part two of this thesis and will not be mentioned further at this point.

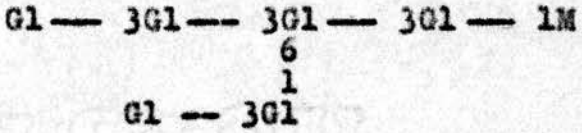
Fucoidin is a heavily sulphated fucose containing polymer (4). The high sulphate content (ca. 33%) corresponds to one sulphate ester group for every fucose unit. The results of methylation studies seen in relation to the extreme stability of the sulphate to alkali clearly indicate that the polysaccharide is essentially a 1,2-linked fucan, carrying sulphate ester groupings at C_4.



Fucoidin
(main repeating unit)

Partial hydrolysis studies have provided evidence of some 1,4-linked fucose residues (5), and it is possible that some of the sugar units are disulphated.

Laminarin, the food reserve carbohydrate of the brown algae, occurs in two forms, one "soluble" and the other "insoluble" in cold water. There is little, if any, chemical difference between the two varieties. From a structural viewpoint they both constitute a β-1,3-linked glucan (6) with a small proportion of 1,6-linkages and/or branch-points at position 6 (7). Apart from glucose, mannitol is present to a small extent (ca.2%) (8) and has been shown recently (9) to constitute mono-substituted end groups, blocking a proportion of the reducing glucose residues ~~in the molecule~~.



Laminarin

G : β-glucopyranose residue

M : mannitol residue.

Rhodophyceae.

The polysaccharides of the red seaweeds are characterised by the presence of galactose sulphates and 3,6-anhydrogalactose. It is notable that sometimes both D- and L-galactose occur together in these polymers. In addition to the sulphated galactans some of the red seaweeds contain other sulphate-free

polysaccharides: the mannan from Porphyra sp., the xylan from Rhodymenia palmata and the food reserve glucan, Floridean starch, from Dilsea edulis. Carrageenin, the sulphated galactan of Chondrus crispus, Gigartina stellata and other related algae, appears to be a mixture of polymers, containing both D- and L-galactose, the two major ones having been designated χ- and λ-carrageenin. χ-Carrageenin consists essentially of an alternating chain of 1,4-linked 3,6-anhydro-α-D-galactose and 1,3-linked β-D-galactose-4-sulphate residues (10). λ-Carrageenin is thought to be a highly sulphated α-1,3-linked polygalactose, consisting almost entirely of D-galactose units (11). Evidence has been presented, however, for the existence of a third component of carrageenin. Rees (12) has shown that a sample of "λ-carrageenin" contained galactose-6-sulphate which by alkali treatment was converted into 3,6-anhydro-D-galactose.

Agar. This galactan can be extracted from several varieties of red seaweeds, notably from species of Gelidium and Gracillaria. It is of considerable importance as a gel-forming agent and finds much use in the preparation of media used for culture of micro-organisms. The polysaccharide material is a mixture of at least two different components, named agaro-pectin and agarose (13). The former consists mainly of 1,3-linked β-D-galactose residues with minor quantities of sulphate, glucuronic acid and pyruvic acid, while agarose is built up largely of alternating units of 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose.

Porphyra umbilicalis galactan shows similarities to agar and to carrageenin. It resembles the former in containing 3,6-anhydro-L-galactose and the latter in its content of D- and L-galactose and of sulphate ester groups on C_4 and C_6 of the galactose units. The 6-sulphate groups are predominantly linked to L-galactose residues (14). However, this galactan differs from agar and carrageenin in the presence of 6-O-methylgalactose residues (15) which are also present in the galactans of other species of Porphyra (16) and the related genus Bangia (17). The transformation of the galactose-6-sulphate by alkali or by the action of an enzymic extract of the seaweed with simultaneous formation of 3,6-anhydrogalactose, has been shown to occur (18), and it is thought that this transformation takes place as a part of the metabolism of these galactans, the 6-sulphated component of carrageenin being the biological precursor of the κ-carrageenin.

Chlorophyceae.

As already mentioned, the polysaccharides of the Chlorophyceae have been far less investigated than those of the two preceding groups. But studies in this department on a number of green weeds during recent years have revealed thatalthough they contain polymer carbohydrate material of a very complex nature, and the different genera synthesise different water-soluble sulphated polysaccharides, they all appear to contain a small proportion of a starch-type polysaccharide. It will

be seen in the subsequent discussion that, in so far as they have been investigated, the sulphated polymers correspond on the whole to the botanical classification. A notable exception however is found in Acrosiphonia centralis, a green seaweed, closely related botanically to Cladophora rupestris. In its polysaccharide content it differs from Cladophora but closely resembles species of Ulva and of Enteromorpha.

While the Phaeophyceae and Rhodophyceae metabolise mainly homopolysaccharides, the green seaweed polysaccharides are frequently built up of four or five different monosaccharide units. When, in addition to the neutral sugars, sulphate ester groups and uronic acid residues are present, the structural studies are obviously attended with considerable difficulties. It must be emphasised, however, that what is regarded as a single heteropolysaccharide, may be a mixture of components. Fractionation and purification of green algal extracts have proved exceedingly difficult and in only a few instances have these been successful. Complete removal of contaminating nitrogen-containing substances from the water-soluble extracts has so far never been achieved. Amino sugars have not so far been found in these extracts, and the nitrogen content is probably due to the presence of protein and ribonucleic acids. And since a small part of this protein persistently resists removal, it appears that there must be some kind of carbohydrate-protein linkage present in the polymer.

The presence of sulphate ester groups has always complicated

the study of seaweed polysaccharides. These acidic functions tend to impede the reactions commonly used in carbohydrate chemistry. Therefore it is desirable to remove the sulphate to obtain a product that can be investigated by the classical methods. But the removal of sulphate without hydrolysis of the polysaccharide to its constituent sugars is usually difficult. The sulphate groups are, except in special cases, extremely stable to alkali, and their stability to acids is in general of the same order as that of the glycosidic linkage. If, however, the sulphated polymer contains a certain proportion of uronic acid residues, the presence of the acid stable uronosyl linkages seem to have a stabilising effect on the whole polysaccharide, and it has been found in these circumstances that the sulphate can be removed by treatment with dry methanolic hydrogen chloride without excessive degradation of the polysaccharide.

It should be pointed out that while sulphated water-soluble extracts appear to constitute the major polysaccharide material in such genera as Cladophora, Acrosiphonia and Enteromorpha, this is not always the case. In species of Caulerpa, for example, the structural polysaccharide is a neutral xylan (19), and in species of Codium this is replaced by a mannan (20).

Cladophora rupestris. The major polysaccharide of C.rupestris (21) (22) is a water-soluble material, $[\alpha]_D$+69°, which upon hydrolysis afforded the following sugars in the molar proportions:

L-arabinose	D-galactose	D-xylose	L-rhamnose	D-glucose
3·7	2·8	1·0	0·4	0·2

The contaminating protein (ca.25%) could not be removed by any of the routine methods, but was reduced to ca.7% by treatment with trichloroacetic acid. Apart from the separation of a β-1,3-linked glucan, resembling laminarin, by chloroform extraction of the acetylated material no further fractionation of this extract was achieved. The sulphate ester groups (ca.20%) present in the residual glucose-free polysaccharide proved extremely stable to alkali and could not be removed prior to the structural investigations. This material was subjected to methylation, periodate oxidation including Barry-degradation and partial acid hydrolysis. These experiments provided evidence that a proportion of the xylose and galactose constituted non-reducing end groups, that the rest of the xylose was present as 1,4-linked units or as 4-sulphated xylose end groups and that the rest of the galactose occurred as 1,3-linked galactopyranose and as 1,6-linked galactofuranose. Furthermore the results indicated that arabinose and rhamnose were present as 1,3-linked pyranose residues. However, interpretation of the methylation results was difficult as the product contained 16% sulphate and may not have been fully methylated. Partial acid hydrolysis afforded inter alia an apparently pure disaccharide containing arabinose only and a trisaccharide containing arabinose, xylose and galactose. Neither of these sugars were isolated as crystalline materials

and hence this can only be regarded as tentative evidence that these sugars really occur together in the same polysaccharide molecule. That galactose, arabinose and rhamnose are indeed present in a single molecule was supported by repeated Barry-degradations (22), the residual polysaccharide, although free of xylose still contained the former three sugars together with a high proportion of sulphate. The latter fact indicates that in this polysaccharide the ester sulphate groups must be located on arabinose and galactose. C.rupestris has been shown to contain a starch-type polysaccharide, which can be extracted together with the sulphated material by N sodium hydroxide after mild chlorite treatment (23).

Codium fragile (20) gave on extractions with cold water a polymer, containing mainly arabinose and galactose, and which on purification on DEAE-cellulose afforded a pure sulphated arabinogalactan (ca.5% of dry weight of weed). Alkali treatment of this polysaccharide reduced the sulphate content (12·7%) by ca.4% with simultaneous increase in the content of 3,6-anhydrogalactose, indicating the presence of sulphate ester groups in 6- or 3-position on galactose units. The hot-water extract of the weed gave a positive iodine test, and a pure starch was isolated by precipitation as the iodine complex (ca. 1% of dry weight of weed). Extractions of the residual weed with zinc chloride (24) and with 4% and 20% sodium hydroxide yielded a mannan (ca.20% of dry weight of weed) that on partial enzymic hydrolysis gave β-1,4-linked mannobiose and -triose in

addition to mannose.

The green seaweed Caulerpa filiformis yielded a water-soluble polysaccharide extract (10% of dry weight of weed), different from that of Cladophora and of Codium (19)(25)(26) and containing the sugars (molar proportions):

D-galactose	D-glucose	D-mannose	D-xylose	L-rhamnose
4	14	2	2	0·5

This water-soluble, sulphated material, $[\alpha]_D$+120°, could be fractionated as the borate complex with cetyl trimethyl-ammoniumhydroxide. This reagent precipitated sulphated polymeric material and left a pure glucan (32% of the water-soluble extract) in the supernatant. Periodate oxidation, methylation and enzymic studies on this glucan indicated that it was very similar to the amylopectin fraction of starch. Fractionation experiments (26) on the residual sulphated material indicated that it is probably a mixture of at least two polysaccharides, possibly an arabinogalactan and a xylomannan.

The residual weed after extraction of water-soluble polysaccharides was treated with chlorite followed by extraction with dilute sodium hydroxide at room temperature. In this way a xylan (5% of dry weight of weed), containing 4% of a contaminating glucan, was obtained. Hot aqueous extraction completely purified this from the glucose polymer. Methylation and periodate oxidation experiments revealed this xylose polymer to be a linear β-1,3-linked xylan and the glucan to be a β-1,3-linked polymer.

As previously stated A.centralis (27) is closely related botanically to Cladophora rupestris but differs distinctly in its content of carbohydrate material. The major polysaccharide is a water-soluble extract, $[\alpha]_D$-31°, which yielded upon hydrolysis the following sugars in the molecular proportions:

D-galactose	D-glucose	D-mannose	D-xylose	L-rhamnose
0.1	1.0	0.2	1.6	1.4

Sulphate (7.8%) and uronic anhydride (ca.20%) were present in addition to the neutral sugars. Acetylation, followed by chloroform extraction gave a glucose-rich acetate. De-acetylation of this resulted in a glucose polymer resembling starch in its colour with iodine. That it was indeed an α-1,4-linked glucan, was shown from its rotation and by methylation studies.

Methylation and hydrolysis of the residual polysaccharide yielded 2,3,4-tri-O-methylxylose, 2,3-di-O-methylxylose, 2,3-di-O-methylrhamnose and 2-O-methylrhamnose. From this it may be deduced that the xylose occurs as end groups and as 1,4-linked units, and that the rhamnose is probably 1,4-linked with branch points at C_3. Partial acid hydrolysis of the original extract of A.centralis led to the isolation of a number of oligouronic acids, of which the aldobiouronic acid 4-O-β-D-glucuronosyl-L-rhamnose was separated and characterised.

The major polysaccharide of E.compressa (28), $[\alpha]_D$-49°, was found to resemble that of A.centralis in that it was sulphated, contained the same main sugars and uronic acid and had a

comparable specific rotation. The sulphate content (11·5%) was however higher than that of the latter weed (7·8%), and furthermore the E.compressa aqueous extract appeared to be devoid of the trace quantities of D-galactose and D-mannose found in A.centralis. The molar proportions of the sugars present in E.compressa:

D-glucose	:	D-xylose	:	L-rhamnose
1·0	:	1·3	:	6·4

differ from those of A.centralis in the higher proportion of L-rhamnose, but since a high proportion of this sugar is linked to glucuronic acid in both these polysaccharides, the stability of the uronosyl link to acid hydrolysis makes a true assessment of the rhamnose content very difficult.

A pure starch-type glucan (ca.3% of the water-soluble extract) was separated by iodine precipitation from the above material, but the residual product still contained a significant proportion of glucose. However it no longer gave a positive iodine test and was unattacked by salivary α-amylase. Further fractionation of the starch-free material could not be achieved despite the investigation of a number of different methods.

The polysaccharide was successfully desulphated by treatment with methanolic hydrogen chloride, the sulphate content being reduced from 16% to 0·7% and the desulphated material isolated in 71% yield. The sulphated and desulphated polysaccharide reduced 0·38 and 0·68 moles of periodate for every

sugar unit. The higher reduction of periodate by the desulphated material reveals that additional α-glycol groupings are formed by desulphation. That the molecule is highly branched or contains a large proportion of 1,3-linkages is indicated by the relatively small amount of periodate reduced. A partial acid hydrolysate of the polysaccharide showed chromatographic evidence for the presence of the same aldobiouronic acid, 4-O-β-D-glucuronosyl-L-rhamnose, as that isolated from A.centralis (27), again emphasising the close similarity in the polysaccharides of these algae.

GENERAL METHODS OF INVESTIGATION

Evaporations were carried out under reduced pressure at or below 40°.

Melting points were recorded as micro melting points on Kofler's hot stage microscope.

Optical rotations were measured in a 1 dm. polarimeter tube at room temperature. Water was used as solvent unless otherwise stated.

Ash contents were determined by ignition of samples (ca.50 mg.) in a platinum crucible until constant weight was attained.

Nitrogen determinations were carried out by a semi-micro modification of the Kjeldahl method (32).

Sulphate was estimated by a modification of Jones and Letham's method (33). Carbohydrate sulphate, containing 120 μg. sulphate or more, was hydrolysed with analar concentrated nitric acid (0·5 ml.) in a glass-stoppered pyrex tube at 100° for ca.12 hours. After addition of a few mg. of Analar sodium chloride and evaporation to dryness over a bunsen burner the tube was kept in an oven at 110° for 2-3 hours and then cooled. Water was added to give a concentration of 60-200 μg. sulphate per ml. An aliquot (1·00 ml.) was transferred to a centrifuge tube followed by an equal volume of the reagent, 4-chloro-4'-aminodiphenyl (0·19%) in 0·1N hydrochloric acid, and a trace of

cetyl trimethylammonium hydroxide (cetavlon). After 2 hr. the resultant precipitate was centrifuged off and 1·00 ml. of the supernatant solution was diluted to 100·0 ml. with 0·1N hydrochloric acid. The optical density of this solution was measured at 254 mμ against water in a SP 500 Unikam spectrophotometer. The value obtained was subtracted from the corresponding value of a sulphate-free blank run in the same way. The amount of sulphate present was finally obtained from a calibration curve, prepared by applying the method to known quantities of analar potassium sulphate.

Uronic anhydride was determined by the Anderson modification (34) of the decarboxylation method (35), or by the carbazole method (36).

Small scale acid hydrolysis. The sample (10-20 mg.) was heated at 100° in a sealed glass tube with N sulphuric acid (1-2 ml.) for 4-6 hours. The hydrolysate was neutralised with solid barium carbonate, filtered, the barium-ions removed with Amberlite IR-120(H^+) resin, and the solution was concentrated to a syrup.

Paper Chromatography. Unless otherwise stated, chromatograms were run with control sugars on Whatman No.1 filter paper. Whatman No.3MM paper was employed for preparative separations. Solvent systems used (v/v-proportions):

A. Ethyl acetate : pyridine : water (10 : 4 : 3)

B. Ethyl acetate : acetic acid : formic acid : water (18 : 3 : 1 : 4)

C. n-Butanol : ethanol : water (4 : 1 : 5), upper layer.

D. Methyl ethyl ketone 50% saturated with water

E. Methyl ethyl ketone : acetic acid : water (9 : 1 : 1), saturated with boric acid

F. Ammonium formate buffer (0·04M, pH 4·3) : isopropanol (65 : 35)

G. Ethyl acetate : pyridine : water (40 : 11 : 6)

H. Ethyl acetate : pyridine : acetic acid : water (5 : 5 : 1 : 3)

I. n-Butanol : pyridine : water (6 : 4 : 3).

Sugars were located on chromatograms by means of one of the following reagents:

a. A saturated aqueous solution of aniline oxalate, followed by heating at 120° for ca.5 minutes. This reagent was used unless otherwise stated.

b. A 2% aqueous solution of sodium metaperiodate (4 parts) mixed just before use with 1% potassium permanganate in 2% sodium carbonate solution (1 part). After spraying the paper is dried at room temperature (61).

c. Bromocresol green (0·1%) in ethanol, made weakly alkaline to give a blue-green colour.

d. Azur II (B.D.H.). A mixture of equal quantities of methylene blue and methylene blue sulphone chloride (62). (0·1 g.) dissolved in water (25 ml.) and the solution mixed with acetone (200 ml.) and methanol (900 ml.).

Ionophoresis was carried out on Whatman No.1 paper at 750 volts for ca.5 hours in borate buffer (pH 10). The ionophoretograms were sprayed with reagent (a) containing 5% glacial acetic acid.

Viscosity measurements were carried out in N potassium hydroxide solution, using a modified Ubbelohde viscometer immersed in a thermostated bath (22.5°). The specific viscosity (η sp) is calculated from the time t_o and t (in seconds) required by the solvent and by the polysaccharide solution, respectively to pass through the viscometer: η sp = $t-t_o/t_o$
The intrinsic viscosity $[\eta]$ is defined as : $[\eta] = \lim_{c \to o} \eta sp/C$
Consequently by measuring the η sp at several concentrations the intrinsic viscosity is obtained by extrapolation to zero concentration.

Dialysis of polysaccharide solutions was effected in cellophane tubes against running tap water for 3 days. Toluene was added to prevent microbial action.

Polysaccharide material was, prior to quantitative estimations, vacuum-dried at 60° over phosphorus pentoxide.

Distilled water is always used unless otherwise stated.

PART ONE

THE WATER-SOLUBLE POLY-SACCHARIDE OF THE GREEN SEAWEED ULVA LACTUCA

INTRODUCTION.

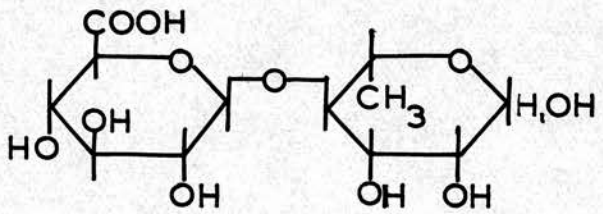
The *Ulva species* belong to the class Ulvaceae, a group of algae occurring frequently along the coastal areas of Northern Europe. A representative of this class is *Ulva lactuca*, commonly called "sea lettuce," indicating its previous use as a culinary dish. It is easily recognised by its broad, delicate leaf-like fronds, which are only two cell-layers thick.

The polysaccharide material of this seaweed was investigated by Brading et al. (29), these authors being the first ones to record the complex chemical composition of the polymer carbohydrates of the green algae. Extraction with dilute sodium carbonate yielded a product containing *ca.* 25% of protein. This was partially removed by the Sevag process (30), although this caused a considerable loss of carbohydrate. The residual material upon hydrolysis afforded the sugars D-glucose (17·7%), D-xylose (9·4%), and L-rhamnose (31%), accompanied by uronic acid (19·2%) and sulphate (16%). The uronic acid was identified as D-glucuronic acid by conversion to the methyl 2,3,4-tri-O-methylglucuronosidamide.

The *Ulva* polysaccharide reduced 1 mol of periodate for every 960 g., indicating that *ca.* every sixth unit contained c-glycol groupings. Removal of the sulphate ester groups with barium hydroxide proved impossible and the polysaccharide was methylated (Haworth reagents) in its sulphated form. The partially methylated product could be fractionated by chloroform extraction, which produced a glucose-rich, nearly sulphate-free

fraction, that was further methylated with Purdie reagents to give a final methoxyl content of 31%. The presence of 2,3,6-tri-O-methylglucose, 2,3-di-O-methylglucose and 2,3,4-tri-O-methylrhamnose was established in the hydrolysate of this product. Paper chromatographic examination of the hydrolysate obtained from the chloroform-insoluble fraction indicated the presence of 2,3,4-tri-O-methylxylose, 2,3-di-O-methylxylose, 2,3,4-tri-O-methylrhamnose and 2,3-di-O-methylrhamnose. Unmethylated xylose and rhamnose were also present, but no trace of free or methylated glucose was detected. It is concluded that xylose or rhamnose are sulphated since the sulphate remained in the chloroform-insoluble fraction. Furthermore the methylation studies indicate that both xylose and rhamnose occur as end groups and as 1,4-linked residues.

Recently McKinnell and Percival (31) have isolated an aldobiouronic acid from a partial hydrolysate of the water-soluble polysaccharide of U.lactuca and characterised it as 4-O-β-D-glucuronosyl-L-rhamnose.



4-O-β-D-Glucuronosyl-L-rhamnose

Esterification and reduction of this acid to the disaccharide glycoside, followed by hydrolysis and characterisation of the derived glucose and rhamnose, confirmed that it comprised D-glucuronic acid and L-rhamnose. Periodate oxidation and methylation of the derived methyl D-glucosyl-L-rhamnoside provided proof that the aldobiouronic acid is the same as that reported present in similar hydrolysates of the polysaccharides from A.centralis (27) and E.compressa (28).

The fractionation of a pure starch from the Ulva lactuca water-soluble extract and further studies on the residual sulphated polysaccharide form the subject matter of the first part of this thesis.

EXPERIMENTAL SECTION

EXPERIMENTAL.

Two batches of the green seaweed Ulva lactuca were obtained: 1. From Nova Scotia, Canada. Collected in July 1960 (250 g. dry weight). 2. From St. Abbs, Scotland. Collected in May 1961 (360 g. dry weight). Each sample yielded approximately the same polysaccharide material. The two batches were pre-treated and extracted in the same way; therefore the extraction of the first batch only will be described in detail.

Expt.1. Extraction of Water-Soluble Polysaccharide.

The seaweed (250 g. dry weight) was moistened with water to bring it into a swollen state and then completely covered with acetone, containing 25% of dimethylsulphoxide. The mixture was kept at ca.30° for 3 days under occasional stirring. The coloured liquid was discarded and the procedure repeated with fresh solvent. This treatment removed most of the colouring matter and other low-molecular compounds such as lipids, free sugars etc. The decolorised seaweed was subjected to four cold-water extractions, each extraction being carried out with 4 litres of water for 14 hours under stirring. The residual weed was finally extracted twice under similar conditions, but with hot water on a boiling water bath and under nitrogen. After centrifugation, filtration, concentration and dialysis the extracts were isolated by freeze-drying as a nearly white, porous solid.

Yield:

1st Cold water extract	10·5 g.	
2nd " " "	12 g.	
3rd & 4th " " "	9 g.	
1st Hot water extract	16·5 g.	
2nd " " "	3·5 g.	
Total	51·5 g.	(20·6%)

Expt.2. Examination of the Polysaccharide Extracts.

Hydrolysis of each of the extracts obtained, followed by paper chromatography in solvents A and B revealed in all cases spots corresponding to glucose, xylose, rhamnose and mannose (trace), the rhamnose spot invariably being the strongest. Using the spray reagent (c), acid spots showed up, the main one moving at the speed of the aldobiouronic acid 4-O-β-D-glucuronosyl-L-rhamnose (31). The extracts had the rotations and percentage composition recorded in Table I.

Table I.

	$[\alpha]_D$	Ash	Nitrogen	Sulphate	Uronic anhydride b)
1st Cold water extract	-48°	10·0	0·74	15·9	15·9
2nd " " "	-50°	13·0	0·50	15·8	15·5
3rd & 4th " " "	-49°	10·0	0·78	13·8	15·2
1st Hot water extract	-45° a)	11·3	1·55	12·9	11·6
2nd " " "	-42° a)	5·9	2·00	10·1	10·5

a) Solution turbid, possibly not correct value b) By decarboxylation.

The presence of a starch-type polysaccharide was indicated, as aqueous solutions of both the cold and hot water extracts gave a blue colour with iodine.

Expt.3. Attempted Removal of Protein (37).

To the 2nd hot water extract, (N-content 2%) (1 g.) in water (100 ml.) was added trichloroacetic acid (4 g.) with stirring. After standing at room temperature for 3 days the precipitated material was centrifuged off, and the polysaccharide in the supernatant solution was recovered by freeze-drying after dialysis and concentration. Yield 0·75 g. (75%), N-content 1·4%.

Expt.4. Equivalent Weight Determination.

The polysaccharide (3rd and 4th cold water extracts) was converted to the free acid form by passing a solution (1 g. in 150 ml. water) four times through a column of Amberlite IR-120(H^+). The resultant acid solution was concentrated and freeze-dried to a white powder (0·77 g.). (Sulphate, 15·3%. Uronic anhydride 18·8% by decarboxylation). Titration of aqueous solutions of this acid polysaccharide (30 mg. portions in 5 ml. water) with 0·01ON sodium hydroxide gave an average equivalent weight of 355.

Expt.5. Treatment of the Polysaccharide Extract with Salivary α-Amylase.

To the polysaccharide (0·1 g.) in water (50 ml.) were added a fresh aqueous solution of salivary α-amylase, sodium chloride (20 mg.) and a few drops of toluene. The digest was incubated

at 35° and after 24 and 48 hours aliquots were withdrawn, which in both cases showed a negative iodine test. The enzyme digest was heated to 70-80° for 5 minutes to denaturate protein, the faint precipitate removed at the centrifuge and the residual polysaccharide recovered by freeze-drying after dialysis, (0.058 g.). $[\alpha]_D$-71° (c, 0.58). Chromatographic examination of the hydrolysate of this starch-free polysaccharide showed the presence of xylose, rhamnose and mannose (trace) in the same proportions as before, while the amount of glucose had been considerably reduced.

Expt.6. Isolation of a Starch-type Glucan from the Polysaccharide Extract (38).

Polysaccharide (10 g.) mixed with celite (2 g.) was dispersed in 0·3% ammonium carbonate solution (500 ml.) and boiled gently under nitrogen for 30 minutes. The suspension was cooled to room temperature, and 20% aqueous sodium chloride (200 ml.) together with iodine (12%) and potassium iodide (20%) in aqueous solution (20 ml.) introduced. After vigorous shaking the mixture was transferred to centrifuge bottles and left for 5 minutes. After centrifugation (ca. 2000 r.p.m. for 10 minutes) the supernatant was decanted off and kept, and the precipitate redispersed in 20% sodium chloride (200 ml.). 0·5N Sodium thiosulphate was added dropwise under rapid stirring until the iodine complex was completely destroyed, excess of the reagent being avoided. To the colourless suspension were added 2N hydrochloric acid (50 ml.)

and iodine-potassium iodide reagent (10 ml.). The reprecipitated starch-iodine complex was centrifuged off as before, the supernatant discarded and the precipitate suspended in 95% ethanol (200 ml.). The dark blue complex was again decolourised with 0·5N sodium thiosulphate and the alcohol concentration adjusted to 70% by addition of water (70 ml.). After a final centrifugation the supernatant was again discarded. The starch was extracted from the celite with hot water under nitrogen and filtered. It was isolated by freeze-drying after dialysis and concentration. Yield 0·12 g. (1·2%). Chromatographic analysis of an acid hydrolysate of this material gave a single spot with a mobility identical with that of glucose.

The supernatant solution obtained from the first centrifugation, containing the residual water-soluble extract was dialysed and the polysaccharide isolated by freeze-drying, 8·3 g. (83%), $[\alpha]_D$-70·7°, sulphate, 19%. The starch-free polysaccharide, hereafter called polysaccharide A, was used for the following experiments unless otherwise stated.

Expt.7. Fractionation Experiments.

It appeared probable that polysaccharide A still contained more than a single component, and different methods of fractionation were investigated.

(1) Attempted Chromatographic Fractionation (39).

Chromatograms were spotted with 1% aqueous solution of polysaccharide (A), eluted with solvent F for 24 to 48 hours

and, after drying, stained by immersion for 5 minutes in developer (d). Sulphated polysaccharide showed up as long dark blue streaks on a light blue background, and no fractionation was apparent. Alteration of the pH of the buffer to 3·3 or to 5·8 caused no improvement of the separation. Increase in the proportion of isopropanol in the solvent to 45% resulted only in slower movement of the polysaccharide. In a quantitative experiment material A (50 mg.), dissolved in a minimum quantity of water, was applied to the starting line of a 3MM paper (23 x 45 cm.). After irrigation in solvent F for 24 hours and drying, the position of the polysaccharide was located by cutting out side strips which were developed with reagent (d). The front and tail of the long, streaky zone were cut out and eluted with water. Hydrolysates of the two fractions showed on paper chromatography the presence of the same sugar constituents: xylose, rhamnose, glucose and mannose (trace). When the original extract was subjected to paper chromatography in solvent F, the starch did not move and could easily be detected on the starting line of the chromatogram by spraying with $\frac{M}{1000}$ aqueous iodine solution. In a quantitative experiment the extract (100 mg.), applied on 3MM paper, was eluted with solvent F for 24 hours. The polysaccharide-containing sections of the paper were located as described above, cut out, extracted from the filter paper with water, dialysed and freeze-dried. The starch-free fraction (65 mg. $[\alpha]_D$-70°, negative iodine test) on hydrolysis and paper chromatography proved to

contain the same sugars as before, whereas the hydrolysate of the starch-fraction (10 mg.) showed a strong glucose spot together with traces of xylose and rhamnose.

(2) Attempted Fractionation by Ionophoresis.

Ionophoresis on 3MM paper in borate buffer (pH, 10) at 1200 volts for 4 hours was followed by detection of the polysaccharide on the dried paper by immersion in reagent (d). A two inch streak extending from the starting line was produced, and no discrete spots were visible.

(3) Attempted Fractionation with Barium Hydroxide (26) (40).

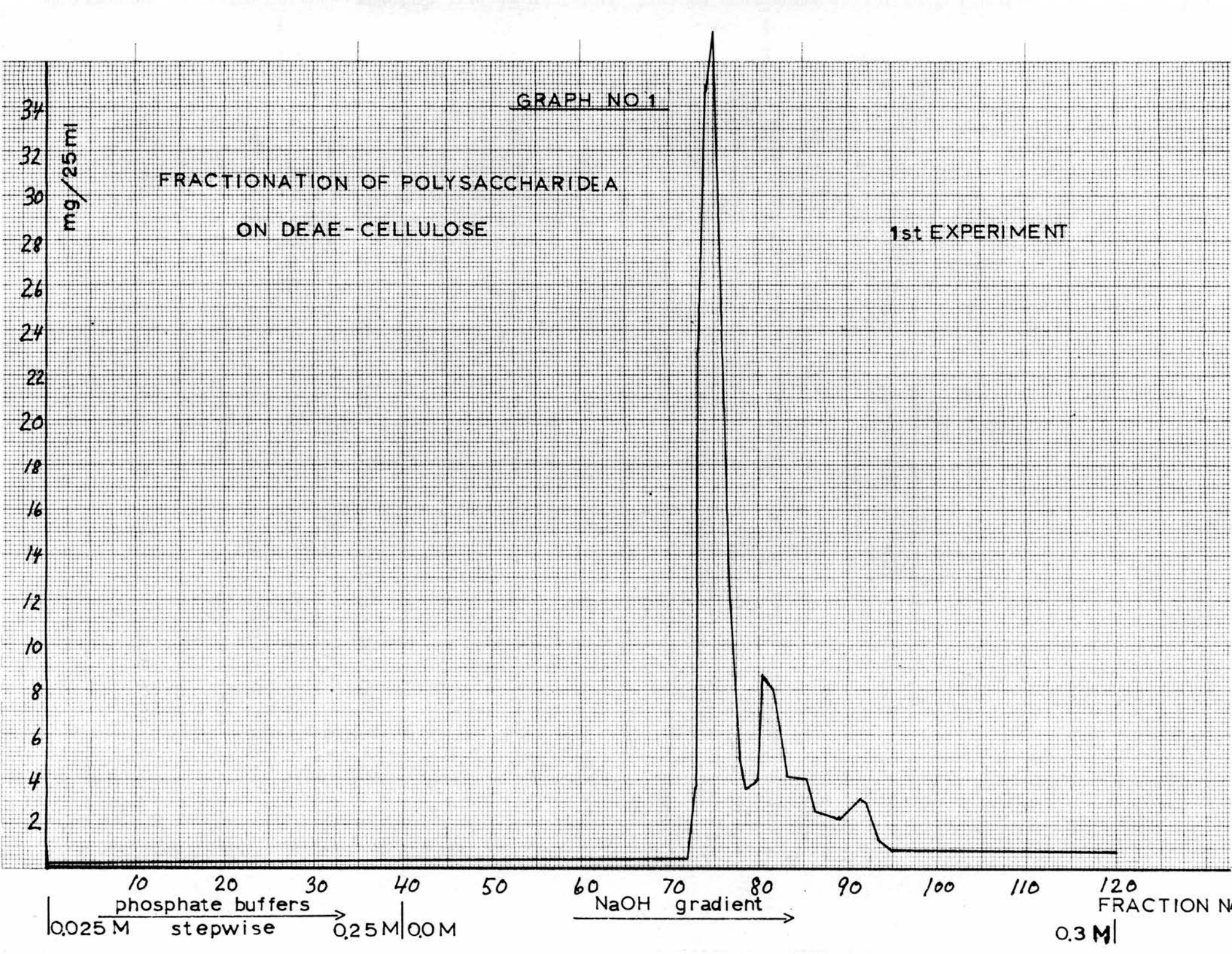
The polysaccharide A (1·5 g.) in 1% aqueous solution was converted to the free acid form by stirring with Amberlite IR-120(H^+) (100 g.). Freeze-drying of the concentrated filtrate gave a white solid (1 g.). This was dissolved in water (1000 ml.) and a saturated solution of barium hydroxide carefully added from a burette. After addition of 27 ml. the mixture became slightly alkaline (litmus) and was left at 2° for 14 hours. The faint precipitate formed was filtered off and proved to consist of barium carbonate. The filtrate was adjusted to pH 5, concentrated, dialysed and freeze-dried (1 g.). Hydrolysis and paper chromatographic examination of this material revealed the presence of xylose, rhamnose, glucose and trace of mannose in approximately the same proportions as before.

(4) Attempted Fractionation by Extraction with Dimethyl Sulphoxide (41).

Polysaccharide A (1 g.) was stirred with dimethyl sulphoxide (50 ml.) at ca.30° for 16 hours. The undissolved material (0·74 g.) was separated on the centrifuge and addition of acetone (250 ml.) to the supernatant gave a fine precipitate (0·21 g.). Hydrolysis and subsequent paper chromatography of the two fractions showed the presence of the same sugars, xylose, rhamnose, glucose and mannose (trace) in the same relative proportions.

(5) Attempted Fractionation on DEAE-cellulose (42).

(i) Prior to preparation of the column the DEAE-cellulose powder (30 g.) was washed alternatively, 3 times each, with 0·5N hydrochloric acid (500 ml.) and 0·5N sodium hydroxide (500 ml.). The washings were carried out in centrifuge bottles and the material was centrifuged off after 15 minutes stirring with acid or alkali respectively. The last washing being done with alkali, the DEAE-cellulose as the free base (in its OH^- form), was suspended in water and filtered off by suction. The material was re-suspended in water as a slurry and poured into a column with a perforated porcelain disc covered with a layer of glass wool in the bottom. The cellulose was allowed to settle by gravity. It was washed with more water until its eluate gave a negative test for chloride. The column (45 x 2·8 cm.) was then converted into the phosphate form by irrigation with 0·5M sodium dihydrogen phosphate (500 ml.), adjusted to pH 6 with sodium hydroxide. Subsequent washing with water


GRAPH NO 1
FRACTIONATION OF POLYSACCHARIDEA
ON DEAE-CELLULOSE
1st EXPERIMENT
mg/25ml
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
10
20
30
40
50
60
70
80
90
100
110
120
phosphate buffers
0.025 M
stepwise
0.25 M
0.0 M
NaOH gradient
FRACTION N
0.3 M

was carried out until the eluate gave a negative test for phosphate ions. Polysaccharide A (0·3 g.) dissolved in water (8 ml.) was applied to the top of the column, allowed to drain into it and then left for 2 hours before elution was started. Stepwise elution was carried out with phosphate buffers, pH 6, (250 ml.) of increasing concentrations, I, 0·025M; II, 0·05M; III, 0·10M; and IV, 0·25M. This technique was followed by gradient elution with sodium hydroxide, (water → 0·3N sodium hydroxide, 1000 ml. each). The eluate was collected in 25 ml. fractions on an automatic turntable, the flow rate being adjusted throughout the elution as it tended to slow down with increasing alkali concentration. The fractions were analysed for content of carbohydrate material by the anthrone method (43), (see graph No.1, p. 34). The polysaccharide material of the two peaks was isolated by freeze-drying after exhaustive dialysis (1 week) to remove phosphate ions. The low yield as well as the low optical rotation and sulphate content indicated that considerable degradation of the carbohydrate had occurred (Table II).

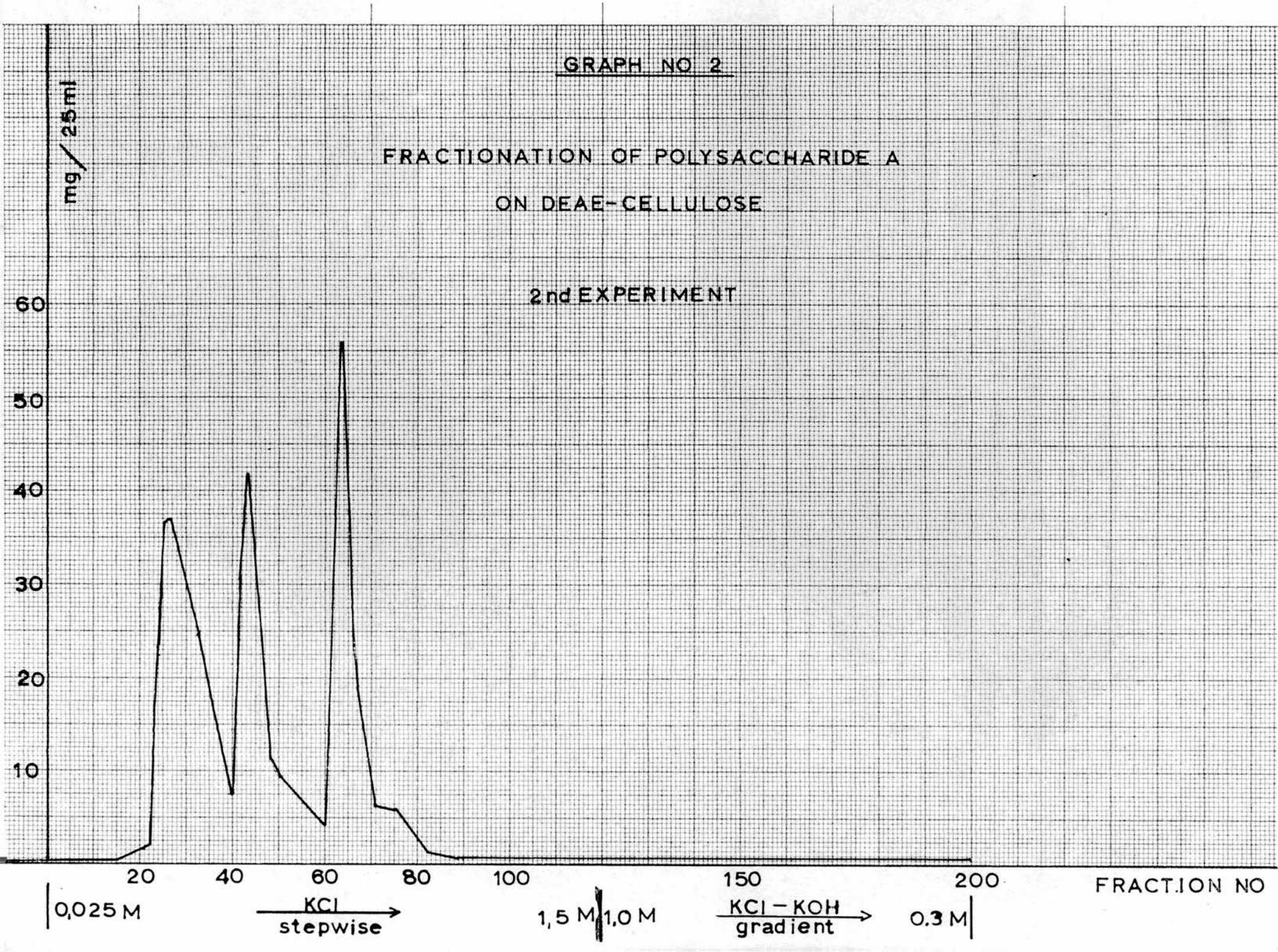
TABLE II.

Peak	Yield, mg.	$[\alpha]_D$	% Sulphate
1	130	-57°	10·6
2	30	-	9·0

(ii) A new and larger column was prepared from a batch of fresh DEAE-cellulose powder. The DEAE-cellulose (60 g.) was subjected to the same pre-treatment as described in the previous experiment. This time however the column (60 x 3·5 cm.) was converted to the chloride form by elution of the free base with 0·5M potassium chloride (500 ml.) followed by washing with water until a negative reaction for chloride was attained. Polysaccharide A (1 g.) in water solution (30 ml.) was allowed to drain into the column which was then left for 2 hours. Stepwise elution was carried out first with potassium chloride solutions of increasing concentrations:

0·025M KCl - 250 ml.
0·05M KCl - 250 ml.
0·10M KCl - 500 ml.
0·25M KCl - 500 ml.
0·50M KCl - 500 ml.
1·0M KCl - 500 ml.
1·5M KCl - 500 ml.

This was followed by gradient elution with M potassium chloride $\longrightarrow$ 0·3M potassium hydroxide (1000 ml. each). The eluate was collected in 25 ml. fractions, and the polysaccharide content of each tube estimated by the phenol-sulphuric acid method (44). The contents of the tubes corresponding to the respective peaks were combined and dialysed. The yields after concentration and freeze-drying were:


GRAPH NO 2
FRACTIONATION OF POLYSACCHARIDE A
ON DEAE-CELLULOSE
2nd EXPERIMENT
mg / 25 ml
60
50
40
30
20
10
20
40
60
80
100
150
200
FRACTION NO
0,025 M
KCl
stepwise
1,5 M
1,0 M
KCl – KOH
gradient
0.3 M

1st Fraction (tube No.24-35) 0·25 g.

2nd " (tube No.42-47) 0·15 g.

3rd " (tube No.62-68) 0·16 g.

It appears from graph No.2 (p. 36) that all the polysaccharide was eluted with the neutral potassium chloride solutions at concentrations below molar. A portion (20 mg.) of each of the three fractions was hydrolysed and subjected to paper chromatographic analysis. Xylose, rhamnose, glucose, mannose (trace) as well as uronic acid were present in all three fractions in apparently the same relative proportions (visual examination). The rotation, the sulphate content and the intrinsic viscosity were determined for each of the fractions (Table III).

TABLE III.

	$[\alpha]_D$	% Sulphate	$[\eta]$
1st Fraction	-67°	16·4	1·50
2nd "	-82°	17·9	1·10
3rd "	-75°	17·9	0·95

The three fractions were shown to give identical infra red spectra, confirming their essential similarity.

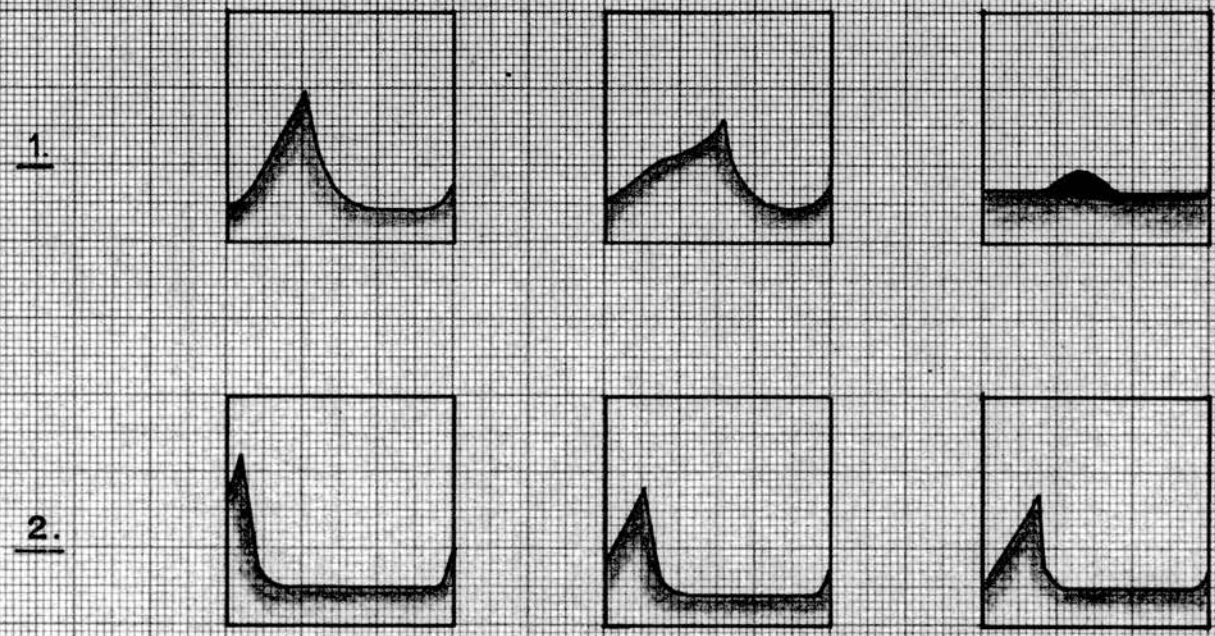
Sedimentation experiments in the ultracentrifuge were carried out on polysaccharide A and on fraction 3 from the DEAE-cellulose column. Solutions of the polysaccharides

GRAPH NO 3

SEDIMENTATION PATTERNS FROM ULTRACENTRIFUGATION

1. POLYSACCHARIDE A

2. 3rd PEAK FROM DEAE CELLULOSE COLUMN



(1·0 ml., containing 0·5 g. in 100 ml. of 0·5M sodium chloride) were examined in a Spinco Model E Analytical Ultracentrifuge at a speed of about 50,000 r.p.m. The sedimentation patterns are shown on graph No.3 (p.37)

Expt.8. Qualitative Tests on Polysaccharide A.

(1) Ester/Lactone (45).

To the polysaccharide (20 mg.) in water solution (1 ml.) were added M hydroxylamine hydrochloride solution (1 ml.) and 2M sodium hydroxide solution (1 ml.). The mixture was heated carefully to the boiling point and then cooled to room temperature. Addition of 2M hydrochloric acid (1·5 ml.) and 1% ferric chloride in 0·1M hydrochloric acid (1 ml.) produced no red colour, indicating that ester/lactone groupings were absent in the polysaccharide.

(2) Keto sugar.

When a hydrolysate of the polysaccharide was subjected to the Seliwanoff test (46), no immediate red colour was formed. On standing over night a faint pink colour developed.

(3) 3,6-Anhydro sugar (47).

The polysaccharide (ca.5 mg.) dissolved in water (2 ml.) was heated at 80° for 10 minutes with a reagent (10 ml.) containing resorcinol (1 mg.) in ethanol (1 ml.) and concentrated hydrochloric acid (9 ml.). No red colour was produced,

indicating the absence of 3,6-anhydro sugar.

(4) Amino sugar (i.e. 2-amino-2-deoxy sugar) (48).

The polysaccharide (0·15 g.) was hydrolysed with 1,5N sulphuric acid (4 ml.) in a sealed tube at 100° for 20 hours. After neutralisation (barium carbonate), filtration and concentration an aliquot of the hydrolysate in water (1 ml.) was mixed with the acetyl acetone reagent (1 ml.) and heated in a boiling water bath for 15 minutes. After cooling ethanol (2 ml.) and the p.dimethylaminobenzaldehyde reagent (1 ml.) were added and the contents mixed well. A bright red colour was formed, being of the same tone as that produced by authentic glucosamine when treated under the same conditions. Several amino acids, however, interfere with this reaction, and as the polysaccharide extract contains a small amount of contaminating protein, a modified Elson-Morgan procedure was used (49) in which amino acids do not interfere. Semi-quantitative estimations using this modification still gave a positive reaction and indicated the presence of less than 0·2% of 2-amino-2-deoxy sugar in the cold water extract and less than 1% in the hot water extract of U.lactuca. Attempts to detect this sugar by paper chromatography (solvents B and H) were unsuccessful. In view of the small proportions present it was thought that the amino sugar might have been derived from the tiny sand hoppers which are difficult to remove completely from the dried fronds of Ulva. Some of these small animals were collected

from a fresh batch of U.lactuca and two portions (each of 0·3 g. dry weight) were extracted for 16 hours with cold and hot water respectively. The extracts were dialysed, concentrated to dryness and hydrolysed with 1·3N sulphuric acid for 18 hours. The neutralised hydrolysates were subjected to the modified Elson-Morgan test and to paper chromatography. The hot water extract gave a strongly positive Elson-Morgan reaction, and the cold water extract gave a weak but still positive reaction. Paper chromatograms after rather heavy spotting were run in solvent B and sprayed with ninhydrin as well as with aniline oxalate. Spots corresponding to 2-amino-2-deoxy-D-glucose (run as control) showed up with both spray reagents.

Expt.9. Estimation of the Relative Molecular Proportions of the Sugars in Polysaccharide A (50).

A standard solution of control sugars was prepared by weighing out milli-molar quantities of xylose, rhamnose and glucose followed by dilution to 50 ml. in a standard flask. Accurately measured volumes containing varying amounts of this solution were applied to the starting line of a number of paper chromatograms and eluted for 16-18 hours in solvent (A). After thorough air-drying the chromatograms were sprayed with a freshly prepared solution of aniline hydrogen phthalate (phthalic acid (1·66 g.) and redistilled aniline (0·91 ml.) dissolved in n-butanol (48 ml.), ether (48 ml.) and water (4 ml.)) and developed at 120° for 5 minutes. The coloured

spots were cut out, transferred to test tubes (2 x 15 cm.) and eluted with 0·7N hydrochloric acid in 80% ethanol (5 ml.) for one hour. The optical density of the solutions was measured in a Unicam spectrophotometer S.P.500 at 360mμ for xylose and at 390mμ for glucose and rhamnose. The readings were made against a blank prepared by eluting a similar piece of the chromatogram which was free from sugars. A linear relationship was found on plotting optical density against weight of each sugar, and from the calibration curves thus obtained the weights of the sugars in a polysaccharide hydrolysate could be read directly from the graph.

For example:

	Optical density	μg of sugar	Approximate molar proportions
Glucose	0·086	28	1
Xylose	0·372	80	3·4
Rhamnose	0·536	116	4·5

Several estimations were carried out and the average molar proportions found to be:

TABLE IV.

Time of hydrolysis, hours	6	16
Glucose	1	1
Xylose	3·4	3·5
Rhamnose	4·8	6·9

Expt.10. Removal of Sulphate from Polysaccharide A.

(1) (i) Desulphation with Lithium Aluminium Hydride.

The acid form of the polysaccharide (0·37 g.) (after storage for 12 hours over phosphorous pentoxide; longer storage led to degradation) was shaken with pure, dry dioxan (80 ml.) for 0·5 hour. Lithium Aluminium Hydride (L.A.H.) (0·4 g.) was added and the mixture refluxed for 14 hours. Then a further 0·2 g. of L.A.H. was added and the heating continued for a further 9 hours. After cooling excess L.A.H. was destroyed with ethyl acetate, hydrochloric acid was added to pH 5 and the mixture vigorously agitated. The greyish precipitate was removed by filtration, water was added to the filtrate followed by evaporation to remove most of the dioxan. The solution (ca.100 ml.) was dialysed against distilled water, changed at intervals. The dialysates were combined and concentrated, giving a solution which showed a strong sulphate reaction (barium chloride). The solution from the dialysis sac was concentrated (10 ml.), and ethanol (100 ml.) added. The derived precipitate after filtration and drying weighed 55 mg. (Yield: 15%. Sulphate: 6·5%. Sulphate in starting material: 19%). Hydrolysis and paper chromatography of the reduced polysaccharide showed the presence of xylose, rhamnose and glucose together with a trace of uronic acid.

(ii) In a second experiment the acid form of the polysaccharide (0·7 g.) was refluxed with L.A.H. (0·7 g.) for 48 hours in dioxan. The reaction mixture was neutralised with

dilute sulphuric acid and filtered. Sulphate in solution was precipitated with barium hydroxide, and after filtration excess barium was removed with Amberlite IR-120(H^+). The resulting solution was concentrated and polysaccharide (15 mg. ca.2%) was precipitated with ethanol (sulphate, nil.) Concentration of the supernatant solution gave a syrup (0·13 g.). Paper chromatography of this syrup in solvent E and spraying with developer (b) revealed two strong spots corresponding to L-rhamnitol and ethylene glycol (run as controls) together with a number of faint spots. Another strong spot also appeared at the point of application. This latter material was separated from the remainder of the syrup on Whatman No.17 paper (Solvent E) and obtained as a white solid (15 mg. Sulphate: nil %). Paper chromatography after acid hydrolysis showed the presence of glucose (strongest spot), xylose and rhamnose, but no trace of uronic acid.

(2). Auto-desulphation.

The acid form of the polysaccharide (1·72 g., $[\alpha]_D$-80° sulphate 19%) was shaken with dry methanol (100 ml.) at room temperature for 30 hours. The residual material was centrifuged off, washed with methanol and isolated by filtration on a G-4 glass filter. After dissolution in water and dialysis the polysaccharide (B) was isolated by freeze-drying (0·91 g., 53%). It had $[\alpha]_D$-55·9° (c, 0·2) and sulphate 2·4%. The supernatant solution from the centrifugation was concentrated to a syrup (0·70 g.). An aliquot was hydrolysed and subjected to paper

chromatography. Spots corresponding to glucose (weak), xylose (strong) and rhamnose (very strong) were obtained. Hydrolysis of the residual, desulphated polysaccharide and subsequent chromatographic examination showed the presence of glucose, xylose and rhamnose in approximately the same amounts. The relative molecular proportions of the sugars in the two hydrolysates were estimated (50) as described previously:

	Glucose	Xylose	Rhamnose
Methanol-soluble material	1	6·3	14·8
Desulphated polysaccharide B	1	1·2	1·5

(3). Desulphation with Methanolic Hydrogen Chloride (51).

The neutral polysaccharide (1·12 g., $[\alpha]_D$-70·7° Sulphate 18%) was shaken with 0·09 M-methanolic hydrogen chloride (150 ml.) at room temperature for 48 hours. Undissolved material was centrifuged off, and the supernatant, after neutralisation (silver carbonate) gave a mixture of syrupy glycosides (80 mg.). Paper chromatography after acid hydrolysis revealed spots of glucose (trace), xylose, rhamnose and uronic acid.

The insoluble polysaccharide was washed with methanol and isolated by filtration. The product was dissolved in water, dialysed and freeze-dried. (Yield: 0·78 g., 70%. Sulphate: 3·8%). Repeated treatment of this product with 0·09M methanolic hydrogen chloride (shaking for 48 hours) gave a 69% yield of

polysaccharide C with a sulphate content of 0·3%. (Overall yield ca.50%). Increasing the strength of the methanolic hydrogen chloride to 0·15M resulted after a single similar treatment in a 68% yield of polysaccharide, $[\alpha]_D$-80°; sulphate content 2·8%. In a second experiment at 14-16° polysaccharide C' (sulphate 5%) was obtained.

(4). Alkali Treatment of the Starch-containing Polysaccharide (52).

To the water-soluble starch-containing polysaccharide (3·0 g., $[\alpha]_D$-49° sulphate 14·1%) dissolved in water (500 ml.), sodium borohydride (0·4 g.) was added and the solution stored for 48 hours at room temperature. Sodium hydroxide (20 g.) and sodium borohydride (3 g.) were then added to the mixture and the loosely stoppered flask was maintained at 82°±2°. After 4 hours a further 3 g. of sodium borohydride were added, and after 10 hours the solution was cooled and made slightly acid with concentrated hydrochloric acid (60 ml.). The mixture was then dialysed until free from chloride ions (3 days), concentrated and freeze-dried to a white solid, polysaccharide D (2·15 g.; 71%) with $[\alpha]_D$-68° (c, 0·54) and sulphate 12·5%. The fall in specific rotation (-49°→-68°) was not due to decomposition of the starch, since the original and the alkali-treated polysaccharides gave blue colours with iodine, of similar intensity. Paper chromatography of an acid hydrolysate of the alkali treated material D revealed spots corresponding to

xylose, rhamnose, glucose, mannose (trace) and uronic acid. In addition two new spots (both pink with aniline oxalate spray) with the mobilities of arabinose and lyxose (trace) were detected in the solvent systems A, B, H and I.

Expt.11. Separation and Characterisation of the Component Sugars of the Alkali Treated Polysaccharide (D).

Polysaccharide (D), (3·0 g.), was hydrolysed with N-sulphuric acid (100 ml.) for 6 hours at 100°. Neutralisation (barium hydroxide) and barium carbonate, filtration, deionisation with Amberlite IR 120(H^+) and concentration gave a syrup (2·35 g.). The syrup was applied to the top of a column (4 x 45 cm.), containing Amberlite CG-45 resin in the acetate form. The neutral sugars were eluted with water until the eluate gave a negative or nearly negative phenol-sulphuric acid test (1·5 litres). This eluate was concentrated to a syrup (1·47 g. after drying over phosphorous pentoxide in vacuo).

The acid fraction was eluted from the column with 10% acetic acid (2 litres). The eluate was concentrated, and the acetic acid removed by repeated additions of water and subsequent evaporations. The final uronic acid containing solution was neutralised with barium carbonate, filtered, concentrated and vacuum-dried over phosphorous pentoxide to a light brown solid (0·93 g.). Paper chromatography (solvent B) of this fraction, after conversion to the free acid with Amberlite IR-120(H^+) resin revealed acid spots only (reagents (a) and (c)); the main spot moving at the speed of 4-O-β-D-

glucuronosyl-L-rhamnose.

The mixture of neutral sugars on paper chromatography (solvent A) gave spots corresponding to xylose, rhamnose, glucose, mannose (trace), arabinose and lyxose (trace). Attempts to separate this mixture on No.17 paper were unsuccessful, and No.3MM paper (ca. twenty sheets, 23 x 45 cm.) was used (solvent A). This gave a satisfactory separation of xylose, rhamnose, glucose and lyxose, while arabinose and mannose had to be rechromatographed in solvent B. The positions of the sugars were located by means of control strips and the respective fractions cut out and eluted from the paper with water until the eluate gave a negative phenol-sulphuric acid test. Subsequent treatment with charcoal, filtration and concentration of these solutions produced the sugar components as separate entities. The glucose fraction was not isolated as it had arisen from two sources: the starch as well as the water-soluble sulphated polysaccharide. To the other fractions, in syrup form, were added a few drops of ethanol. Rhamnose crystallised readily, xylose and arabinose after some days.

L-Rhamnose hydrate (275 mg.), m.p. and mixed m.p. 80-90°. $[\alpha]_D$ +9·0° (c, 1·09).
m.p. and mixed m.p. after removal of hydrate water: 122-123°, derived benzoylhydrazone (53): m.p. and mixed m.p. 185-189° (decomp.).

D-Xylose (190 mg.), m.p. and mixed m.p. 144-145°. $[\alpha]_D$ +18·3° (c, 1·01).

D-Arabinose (18 mg.) m.p. and mixed m.p. 156-158°
$[\alpha]_D$-100·6° (c, 0·517),
derived benzoylhydrazone (53): m.p. and mixed
m.p. 204-208° (decomp.)
m.p. depressed by admixture with L-arabinose
benzoylhydrazone.

D-Mannose (<5 mg.), chromatographically pure syrup.
Phenylhydrazone (53): m.p. and mixed m.p. 190-192°.

D-Lyxose (<5 mg.), chromatographically and
electrophoretically pure syrup. D-configuration
assumed because of negative rotation,
$[\alpha]_D$ D-lyxose -14°. Attempted formation of
the crystalline phenylosazone (54) was unsuccessful.

D-Glucose was separated as a syrup on 3MM paper from a deionised hydrolysate of the starch-free polysaccharide (A). It had $[\alpha]_D$+48·5° (c, 2·7, found by estimation of reducing power (55)). The derived phenylosazone had m.p. and mixed m.p. 208-210° (decomp.). Incubation of an aliquot with the specific enzyme glucose oxidase at 35° for 24 hours converted the sugar completely into gluconic acid (56), as revealed by paper chromatography in solvent (B) spray reagent (C).

Expt.12. Determination of the Relative Molecular Proportions of the Sugars in Polysaccharide A, B, C and D and in the Original Starch-Containing Extract.

Each of the polysaccharides (50 mg.) was hydrolysed with 1·3N-sulphuric acid (2 ml.) for 5 hours. After neutralisation with barium carbonate, deionisation with Amberlite IR-120(H^+) resin and concentration to dryness the residual syrups were dissolved in ethanol (0·5 ml.). The relative molecular proportions of the sugars present in the respective solutions were estimated by the Wilson-method (50) as described previously. The paper chromatograms were spotted with 10μl. portions of the hydrolysates and then eluted in solvent I for ca.40 hours.

TABLE V.

	Glucose	Xylose	Rhamnose	Arabinose
Polysaccharide A	1·0	3·4	5·0	
" B	1·0	1·0	1·1	
" C	1·0	2·3	3·2	
" D	1·0	1·6	2·0	0·1
Starch-containing extract	1·0	1·9	2·5	

Expt.13. Treatment of Polysaccharide (D) with Sodium Methoxide and subsequent Detection of 2-O-methylxylose after Hydrolysis.

(1) Desulphated polysaccharide (D) (2·0 g.), (dried over phosphorus

pentoxide at 60° in vacuo for one week) was soaked in dry methanol and kept for two days with occasional shaking to remove further traces of water probably present. The dried material was filtered off rapidly by suction and added to a solution of lithium borohydride (0·2 g.) and sodium (6 g.) in dry methanol (250 ml.), after which the suspension was refluxed for 24 hours. The insoluble polysaccharide material was recovered by filtration and after washing with methanol and drying still weighed 2·0 g. This was hydrolysed with N sulphuric acid (60 ml.) at 100° for 4 hours. Neutralisation (barium hydroxide and barium carbonate), deionisation (Amberlite IR-120(H^+) resin) and several additions of methanol with subsequent evaporations to remove the last traces of borate, furnished a syrup, (X; 1·37 g.). This was applied to a number of sheets of 3MM paper and separated in solvent D. In this way a small amount (ca.5 mg.) of chromatographically pure 2-O-methylxylose was isolated. It moved with the speed of authentic 2-O-methylxylose in several solvent systems, and gave spots of the same colour with aniline oxalate spray. When spraying the chromatogram with triphenyltetrazolium chloride (which requires a free C_2-hydroxyl to react) neither the isolated sugar, nor authentic 2-O-methylxylose was revealed, while 3-O-methylxylose and 3-O-methylarabinose readily gave pink spots.

The syrup (X) on paper chromatography showed the presence of several trace spots, possibly corresponding to other methylated

pentoses, but no spot with the mobility of the expected 3-O-methylarabinose was detected.

(2) Demethylation of the 2-O-Methylxylose (57).

2-O-Methylxylose (ca.2 mg.) was dried over phosphorus pentoxide in vacuo for 24 hours. Dry methylene dichloride (2 ml.) was added and the mixture cooled down to -80° (solid carbon dioxide/acetone). Boron trichloride (ca.2 ml.), cooled in the same way, was transferred to the flask which was stoppered and left at -80° for 1 hour, then at room temperature for 24 hours, the whole time under anhydrous conditions. Excess boron trichloride and solvent were removed under diminished pressure, followed by several additions of methanol and subsequent evaporations to remove borate. The residue was finally dissolved in a drop of methanol/water and subjected to paper chromatography in various solvents. In all cases spots corresponding to xylose and 2-O-methylxylose (trace) were revealed.

(3) Attempted Preparation of 2-O-Methylxylose Anilide.

To the residual quantity of 2-O-methylxylose (3-4 mg.), thoroughly dried, was added redistilled aniline (1 drop), absolute ethanol (1 ml.) and the solution refluxed for 0·5 hour with exclusion of moisture. The product on paper chromatography revealed no spot on spraying with aniline oxalate, indicating the absence of reducing sugar. The brownish solution was partly decolourised with a little

charcoal and after filtration the alcohol was allowed to evaporate. A few tiny crystals appeared, but the quantity formed was found to be too small for recrystallisation and was subjected to washing with ether/light petroleum (60-80°). The slightly brownish crystals had m.p. 110-120° (decomp.) undepressed by admixture with an authentic specimen of 2-O-methyl-N-phenylxylosylamine (recorded m.p. 125° (58)).

Expt.14. (1) Periodate Oxidation, (2) Borohydride Reduction, (3) Alkali Treatment, and (4) Hydrolysis of the Starch-containing Polysaccharide.

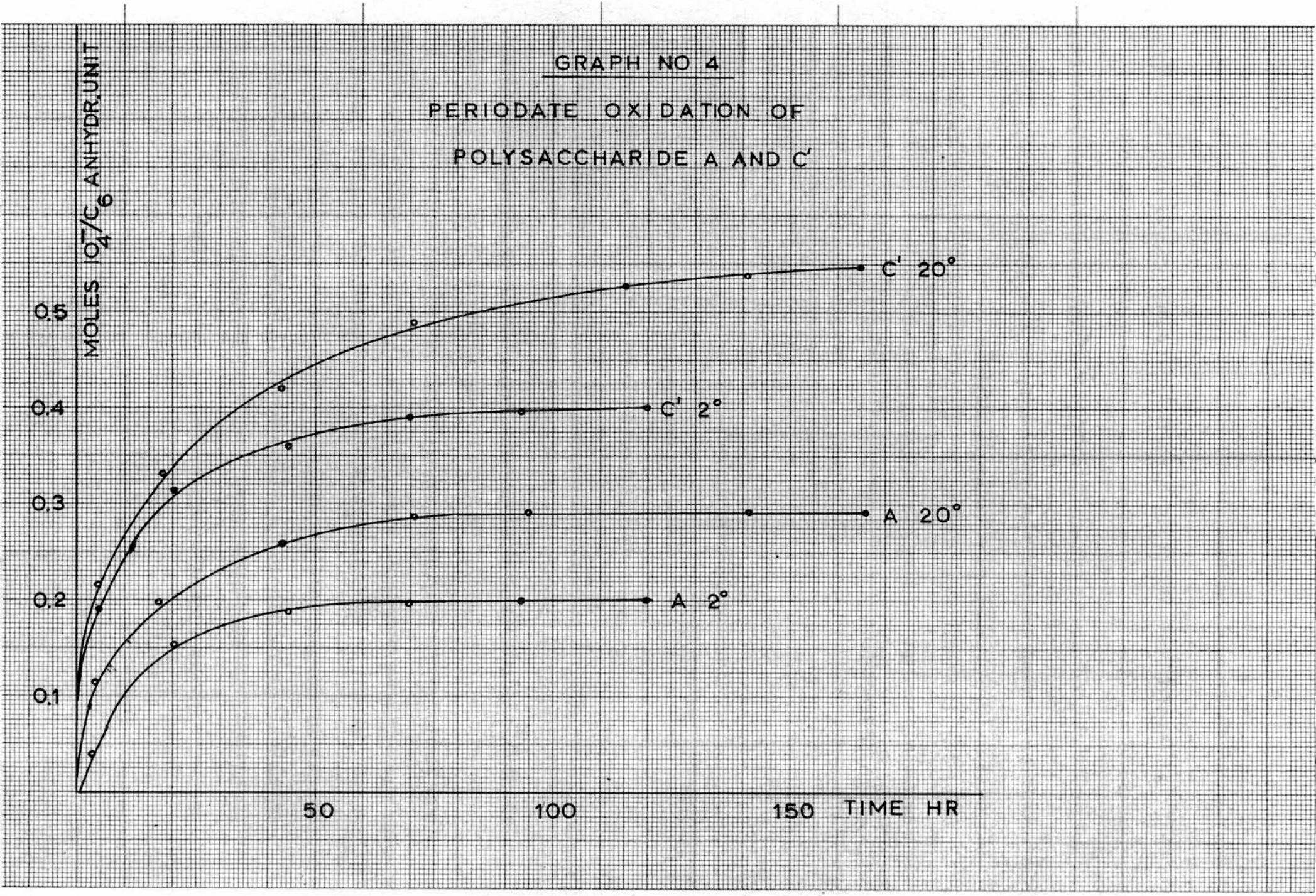
To the starch-containing extract (1 m Mol, 0·16 g.)[X] dissolved in water was added sodium metaperiodate (2.5 m Mol, 0·53 g.) and the volume made up to 50·0 ml. with water. The reaction solution was left at room temperature in the dark and the oxidation process followed spectrophotometrically (59). After ca.100 hours the readings were constant, corresponding to a reduction of 0·54 moles of periodate for every C_6-anhydro unit. The oxidation was stopped by the addition of ethylene glycol, the solution dialysed and then concentrated to ca.25 ml. This was followed by the addition of sodium borohydride (0·10 g.). After 24 hours at room temperature sodium hydroxide (1 g.) and sodium borohydride (0·15 g.) were added and the mixture heated at 80±2° for 6 hours. The cooled solution was neutralised with hydrochloric acid to pH 4-5, dialysed and concentrated to dryness. The residue was hydrolysed with N

[X] 1 m Mol = 1 C_6-anhydro unit

sulphuric acid (4 ml.) for 6 hours at 100°, and the hydrolysate worked up in the usual way. Paper chromatography of the derived syrup showed the presence of xylose, rhamnose, glucose, mannose (trace) and arabinose, but not of lyxose.

Expt.15. Periodate Oxidation of Polysaccharide (A) and (C') (59).

Polysaccharide (C') (sulphate content 5%) was obtained accidentally by desulphation of polysaccharide A with 0·10M methanolic hydrogen chloride at 12-14° instead of at room temperature. Each of the two polysaccharides (1 m Mol, 0·16 g.) was dissolved separately in 0·2M acetate buffer pH 3·6 (25 ml.) (60) and made up to 50·0 ml. in a standard flask with 0·10M sodium metaperiodate dissolved in the same buffer. Thus the final concentration of polysaccharide was 0·02 molar and that of periodate 0·05 molar. The reaction mixture was kept at 2° in the dark. At intervals the reduction of periodate was measured by removing an aliquot (0·10 ml.) of the solution and diluting to 100·0 ml. with water before reading the optical density at 223 mμ against a water blank. The fraction of periodate reduced was then found from a calibration graph obtained by reading the optical density of an identical dilution of a 0·05M sodium meta-periodate and an equimolecular sodium iodate solution under the same conditions. Thus the molar consumption of periodate for every C_6-anhydro unit at different time intervals could be found.


GRAPH NO 4
PERIODATE OXIDATION OF
POLYSACCHARIDE A AND C'
MOLES IO_4^-/C_6 ANHYDR. UNIT
0.5
0.4
0.3
0.2
0.1
C' 20°
C' 2°
A 20°
A 2°
50
100
150
TIME HR

A second periodate oxidation experiment on the polysaccharide (A) and (C') was carried out at room temperature, otherwise using similar conditions as above. (Graph No.4 p 53)

Expt.16. Preparation and Isolation of the Oxo Derivatives of Polysaccharides A, B, C and C'.

The four different polysaccharide materials which were employed had the following sulphate contents:

Polysaccharide	A	Sulphated (18% sulphate)
"	B	Desulphated by shaking the acid form of A with dry methanol (2% sulphate)
"	C	Desulphated twice with methanolic hydrogen chloride (0·3% sulphate)
"	C'	Partly desulphated with methanolic hydrogen chloride (5% sulphate).

Each of the samples (0·5 m Mol, 80 mg.) was oxidised separately with sodium metaperiodate (2·5 m Mol, 0·53 g.) in water (50 ml.) at room temperature. The reaction was followed spectrophotometrically (59) until complete oxidation (80 hours) and excess periodate removed by addition of ethylene glycol. The solutions obtained were titrated with 0.05N sodium hydroxide (micro-burette) to phenolphthalein end point to measure the amount of formic acid produced. From the titration figures attained was subtracted a blank value obtained by titration of an aliquot of polysaccharide A to allow for the acid functions present in the non-oxidised polysaccharide. The oxo-

polysaccharides were isolated from the titrated solutions by freeze-drying after dialysis and concentration.

TABLE VI.

Polysaccharide	A	B	C	C'
Moles of periodate reduced/ C_6-anhydro unit after 80 hr.	0·30	0·65	0·65	0·52
Moles of acid formed/ C_6-anhydro unit after 80 hr.	0·12	0·17	0·17	0·19
Yield of oxopolysaccharide (mg.)	65	55	60	63

Expt.17. Estimation of the Relative Molecular Proportions of the Residual Sugars in the Oxopolysaccharides.

Each of the oxopolysaccharides (A_o), (B_o), (C_o) and (C'_o) (20 mg.) was hydrolysed separately with N-sulphuric acid at 100° for 4 hours. After neutralisation (barium carbonate) treatment with Amberlite IR-120(H^+) resins and concentration syrups were obtained which were dissolved in 0·25 ml. ethanol/water (1:1). Aliquots (0·025 ml.) of the respective hydrolysates were applied to Whatman No.1 papers which were eluted with solvent (A). The dried papers were sprayed with aniline hydrogen phthalate and the proportions of the sugars estimated according to the method of Wilson (50).

TABLE VII

Polysaccharide	Glucose	Xylose	Rhamnose
A_o	1·0	3·3	8·3
B_o	1·0	1·2	1·2
C_o	1·0	2·7	1·8
C_o'	1·0	2·0	3·9

The hydrolysates of B_o, C_o and C_o' gave on paper chromatographic examination in solvent B and spraying with reagent (a) or (c), a spot corresponding to 4-O-glucuronosylrhamnose, the aldobiouronic acid isolated from non-oxidised polysaccharide (31). The hydrolysate of oxo A, however, showed no acid spot.

Expt.18. Uronic Anhydride Estimations of the Oxidised and non-Oxidised Polysaccharides by the Carbazole Method (36).

Separate samples of polysaccharide (10-20 mg., accurately weighed) were dissolved in water and the volume adjusted to 50·0 ml. Aliquots of 2·00 ml. were pipetted out for each test, which were carried out according to the original procedure. As the principle of the method is based on comparison of a colour intensity obtained with that produced by known quantities of uronic acid, a standard graph of pure glucurone was prepared.

The carbazole method is not completely specific for uronic

acids. Therefore attempts were made as follows to eliminate the interference of non-uronic acid constituents.

1. Reduction of the oxopolysaccharides with sodium borohydride prior to the estimations had no significant effect on the results.
2. A mixture of glucose, xylose and rhamnose which was subjected to the estimation had an apparent "uronic anhydride" content of 3·8%.
3. The supposedly uronic acid-free polysaccharides from the green seaweeds Cladophora rupestris and Chaetomorpha capillaris were likewise subjected to the carbazole estimation. According to this the C.rupestris extract contained 4·0% and the Chaetomorpha polysaccharide 2·7% of "uronic anhydride."
4. The method applied to commercial starch gave a "uronic anhydride content" of 9·5%.

The probable explanation of these unexpected results is that the brownish colour formed by the action of strong acid on carbohydrate material absorbs light at the same wave-length as the coloured carbazole-uronic acid product which has its absorption maximum at 520mμ. It was therefore decided to introduce an approximate correction factor of 3·5% in the present experiments.

TABLE VIII

Polysaccharide	% Uronic anhydride according to the method	% Uronic anhydride after correction.
A_o	4.0	0.5
B_o	8.5	5.0
C_o	10.8	7.3
C'_o	6.8	3.3
A	17.6	14.1
B	16.2	12.7
C'	20.5	17.0

Desulphated polysaccharide C was not available for this estimation.

Expt.19. Qualitative Test on Ester/Lactone in the Desulphated Polysaccharides (B) and (C').

(1) Desulphated polysaccharide (40-50 mg.) was subjected to the hydroxylamine-ferric chloride test (45) as described previously (p.37). The polysaccharides (B) and (C') (material C was not available) both gave a bright red colour, indicating the presence of ester/lactone, while polysaccharide A as well as the aldobiouronic acid 4-O-glucuronosylrhamnose, when subjected to the test, gave a negative reaction.

(2) When each of the two desulphated materials (B) and (C') (50 mg.) was shaken with 0·1N sodium hydroxide (2 ml.) for 3 minutes at room temperature and then subjected to the test as above, the reaction was almost completely negative. This was

also the case when authentic glucurone (30 mg.) was treated under the same conditions.

Expt.20. Attempted Detection of Uronic Acid Cleavage Products after (1) Periodate Oxidation, (2) Borohydride Reduction and (3) Acid Hydrolysis of Polysaccharide (A).

(1) Polysaccharide (A) (1 m mol, 0.16 g.) dissolved in 0.2M acetate buffer pH 3.6 (100 ml.) was oxidised with sodium meta-periodate (5 m mol, 1.07 g.) at 2° in the dark for 48 hours. Excess periodate was destroyed by addition of ethylene glycol, the solution dialysed and then concentrated to 30-40 ml. Sodium borohydride (2 m Mol, 80 mg.) was added and the solution left at room temperature for 24 hours, followed by treatment with Amberlite IR-120(H^+) resin and concentration. Methanol was added several times and evaporated off after each addition to remove borate. Finally the residual polyalcohol was hydrolysed with N sulphuric acid (3 ml.) for 4 hours at 100° and the hydrolysate worked up the usual way. Paper chromatographic examination in solvent (A) and (B), using spray reagent (C), revealed no spots corresponding to the expected glyceric or erythronic acid. One acid spot was present however, moving at the rate of the aldobiouronic acid 4-O-glucuronosylrhamnose. The hydrolysate was subjected to rehydrolysis with 2N sulphuric acid for 4 hours at 100°, but the resulting rehydrolysate on paper chromatography gave the same result, although the aldobiouronic acid spot had become

weaker.

(2) A second periodate oxidation was carried out: To the polysaccharide A (2 m Mol, 0·32 g.) dissolved in the same acetate buffer (50 ml.) was added sodium metaperiodate (10 m Mol, 2·14 g.), thus making the concentration of polysaccharide 0·04 molar and that of periodate 0·2 molar. The mixture was left in the dark at 2° for 24 hours. Then an aliquot was withdrawn and hydrolysed after dialysis. Paper chromatography of the hydrolysate clearly showed the presence of uronic acid. The oxidation was then continued at room temperature and followed spectrophotometrically until no more periodate was reduced. Destruction of excess periodate with ethylene glycol and subsequent dialysis was followed by concentration to ca.30 ml. and addition of sodium borohydride (5 m Mol, 0·2 g.). After standing 14 hours at room temperature the mixture was dialysed again, concentrated and subjected to additions of methanol with subsequent evaporations. The residue was hydrolysed and worked up as usual and the resulting hydrolysates examined by paper chromatography in solvent (A), (B) and (C). Neither glyceric nor erythronic acid appeared to be present, while a weak spot was revealed (spray reagent (a) or (c)) corresponding to the same aldobiouronic acid as obtained before.

DISCUSSION.

Extraction of the weed. The supply of seaweed collected in lower Bay of Fundy, Nova Scotia, was kindly presented by Miss McFarlane.

Decolourisation of the dried, whole weed was attempted in different solvents: acetone, aqueous acetone and aqueous and absolute ethanol. Each of these treatments apparently removed only a minor proportion of the colouring matter and was not comparable with the method finally adopted, using acetone and dimethyl sulphoxide (3:1 w/w). Excess acetone was employed to prevent extraction of polysaccharide by the dimethyl sulphoxide (41). The resultant pale greenish off-white seaweed material, after washing with acetone, was dried and subjected to extractions with water. Brading et al. (29) extracted U.lactuca with dilute alkali, obtaining a product rich in protein (4%N). Successive extractions with cold and hot water (Expt.1) however, afforded products of lower nitrogen content (0·5-2·0%) but still with a satisfactory yield of polysaccharide (ca.20% of dry weight of weed). The related green seaweed E.compressa under similar conditions gave a comparable yield (16·8%) of water-soluble polysaccharide (28). The successive extracts obtained were very similar but contained slightly decreasing proportions of sulphate, uronic acid and rhamnose, (the latter being detected by paper chromatography of the respective extract hydrolysates), thus indicating that these three components occur together in the polysaccharide.

As the carbohydrate content of the extract decreased there was a corresponding increase of nitrogen containing material.

Ideally a polysaccharide extract should be completely free of protein, and any other non-carbohydrate material, before analytical and structural work is carried out. The green seaweeds have proved extremely difficult, however, to free from protein. A number of different methods was investigated by Fisher (21) who found trichloroacetic acid (37) the most promising reagent for reducing the nitrogen content in the polysaccharide extract of Cladophora rupestris. It is significant that the trichloroacetic acid treatment when applied to the polysaccharide extracts of Cladophora rupestris, Cauerpa filiformis, E.compressa as well as U.lactuca (2nd hot water extract) (Expt.3) invariably resulted in nearly the same residual percentage of nitrogen (1·4-1·5%), no matter what the initial nitrogen content of the various extracts was. Further reduction of protein by repeated trichloroacetic acid treatment was not achieved (21). This might be regarded as an indication that a proportion of the protein is chemically linked to the polysaccharide.

Equivalent weight. Titration of aqueous solutions of the acid form of the polysaccharide with sodium hydroxide (Expt.4) resulted in an average equivalent weight of 355. The content of uronic anhydride and sulphate in this material was found to be 18·8% and 15·3% respectively. The equivalent weight of uronic anhydride is 176 and of ester linked sulphate it is 96.

Hence every 100 g. of polysaccharide contains 18·8/176 = 0·107 equivalents of uronic anhydride and 15·3/96 = 0·160 equivalents of sulphate. This corresponds to 0·267 equivalents of acid per 100 g. of polysaccharide or an equivalent weight of 100/0·267 = 374. The experimentally found equivalent weight of 355 is in reasonable agreement with, and only 5% lower than, the calculated one of 374.

Isolation of the starch fraction. The presence of a starch in the polysaccharide extract was indicated since it gave a positive iodine test before, but not after treatment with salivary α-amylase (Expt.5). The hydrolysate of the enzyme-treated polysaccharide also showed a weaker glucose spot on paper chromatography than did the hydrolysate of the original polysaccharide. As in the case of E.compressa (28) it proved impossible to remove glucose completely with α-amylase, thus showing that a proportion of this sugar is present as non-starch material. A starch-type glucan can usually be separated from sulphated polysaccharide material by acetylation and chloroform extraction, leaving the sulphated fraction undissolved. But it was considered simpler and less likely to degrade either the starch or the sulphated polysaccharide to use the method of iodine precipitation (38) to separate the starch (Expt.6). This procedure was originally applied only to materials containing 10% or more of starch (38). But it was found that as little as 1% of starch could be satisfactorily separated from other polysaccharide material, the latter being

recovered in good yield (83%). The purity of the glucan was confirmed as an acid hydrolysate on paper chromatography showed a single spot corresponding to glucose.

Fractionation experiments (Expt.7). The starch-free polysaccharide (A) upon hydrolysis afforded xylose, rhamnose, glucose and traces of mannose in addition to uronic acid and sulphate. In view of this heterogeneity polysaccharide A was subjected to a variety of fractionation procedures.

Polysaccharides often occur in nature as complex mixtures and their separation into single entities is one of the major problems of carbohydrate chemistry. Before structural investigations can be carried out careful purification and fractionation procedures must be investigated. There is at present a large number of fractionation methods available, but though they all in certain instances can lead to successful separations none of them is universally applicable. And as far as there does not exist any unambigous way to establish the homogeneity of a polysaccharide it is necessary to try various fundamentally different fractionation methods. If in no case separation is achieved then one must accept as a probability the uniformity of the polymer. In spite of the inability to fractionate the starch-free polysaccharide of E.compressa (28) the corresponding polysaccharide (A) of U.lactuca was subjected to various new techniques. The method of Spolter and Marx (39) employing an isopropanol-water system buffered with formic acid/ammonium formate was

originally developed for the resolution of mixtures of sulphated mucopolysaccharides. It was thought that this system might separate any sulphated uronic acid containing polysaccharides in (A) from neutral components (Expt.7). However, the method proved to be of no value in the present experiments although it was found possible (Expt.7(1)) to separate the original extract into the starch and the charged polysaccharide (A) by this method.

Polysaccharides containing cis α-glycol groupings generally are capable of forming borate complexes. This can be utilised for fractionation purposes, and ionophoresis of polysaccharides in borate buffer on filter paper has in some instances led to separations (63). But it is difficult to carry out these separations on a preparative scale and it often is a problem to detect polysaccharides on cellulose paper. Therefore in some instances it is advantageous to use glass fibre paper recommended by Smith et al. (64). Sulphated polysaccharides, however, are easily detected by immersion in solutions of p.toluidin blue, methylene blue etc. Attempted ionophoretic separation of polysaccharide (A) (Expt.7(2)) was unsuccessful and only resulted in the formation of a streaky zone extending from the point of application.

Barium hydroxide has been used for fractional precipitation of certain hemicelluloses (40), notably those containing mannose with free 2,3-cis-and galactose with free 3,4-cis hydroxyl groups. A partial fractionation was obtained by this

method of the sulphated polysaccharide of Caulerpa filiformis (26). An aqueous solution of the sulphated material of U.lactuca, however, produced no precipitate on addition of saturated barium hydroxide (Expt.7(3)).

Fractionation of hemicelluloses has also been effected by selective extraction with dimethyl sulphoxide (41). An advantage with this neutral solvent is that alkali-labile acetyl groups, frequently present in hemicellulosic materials, are not affected during the extraction. Polysaccharide (A) on extraction with dimethyl sulphoxide yielded a soluble fraction and a residual material (Expt.7(4)). No obvious fractionation had occurred, however, since the same sugars in the same relative proportions were present in both fractions (paper chromatography of hydrolysate).

Quaternary ammonium salts are frequently used in the fractionation of carbohydrate polymers (65)(66). A mixture of acidic and neutral polysaccharides may be separated by precipitation of the acidic components with cetyl trimethylammonium halides or other cationactive quaternary ammonium salts. This technique has been extended (67) to neutral polysaccharides capable of forming borate complexes. It was shown by Lindberg et al. (68) that if the free quaternary base was used instead of the halide, the ionic strength and the alkalinity were kept at a lower level during the fractionation and the unprecipitated material could be more easily recovered. By this method a pure glucan was separated from the sulphated

polysaccharide of the green seaweed Caulerpa filiformis (25). But the sulphated fraction of this polysaccharide as well as that of Cladophora rupestris (21), A.centralis (27) and E.compressa (28) persistently resisted fractionation by the use of this procedure although different modifications were investigated. Consequently in view of the striking similarity between the polysaccharide of U.lactuca and that of A.centralis and E.compressa this method of fractionation was not tried in the present case.

Certain cellulose derivatives with ion exchange properties have recently been utilized for the separation of mixtures of polymers, especially in the field of proteins and polysaccharides. These cellulose derivatives may be of acidic nature such as CM- (carboxymethyl) cellulose or of a basic nature such as DEAE- (diethylaminoethyl) cellulose or ECTEOLA- (epichlorohydrin triethanolamine) cellulose (69). These compounds combine the properties of cellulose powder and ion exchangers and would be expected to be suitable for selective chromatographic separations. Neukom et al. (42) found, analogous to the fractionation of acidic and neutral polysaccharides with quaternary ammonium salts, that acidic polysaccharides were readily adsorbed on anion exchange cellulose of neutral reaction, whereas neutral polysaccharides were not or at most only weakly adsorbed. Thus by using DEAE-cellulose in different forms (phosphate, borate, hydroxyl etc.) and a suitable elution medium with changing pH values and/or

electrolytic concentrations they succeeded in fractionating wheat starch dextrin, a mixture of sugar beet araban and pectic acid, and other mixtures (42). Polysaccharide material from a green seaweed had not previously been subjected to this type of fractionation. The starch-free polysaccharide (A) was applied to a DEAE-cellulose column in the phosphate form according to the conditions described by Neukom et al. (42) (Expt.7(5)) when separating acidic from neutral polysaccharide. The column was eluted with phosphate buffers of the same neutral pH value, but of increasing concentrations (0·025-0·25 molar), in an unsuccessful attempt to separate any neutral polysaccharide. The elution was then continued with alkali of increasing strength (zero - 0·3 molar), and the acidic polysaccharide was eluted in low yield as two separate fractions (see Graph No.1, p.34). The low yield and sulphate content of the fractions, however, indicated that alkaline degradation of the polysaccharide had occurred (Table II, p.34

The elution of an acidic polysaccharide from an anion exchange cellulose column with dilute alkali depends on the fact that the increase in pH gradually depresses the ionisation of the tertiary amine groups of the adsorbent and thus depresses its anion binding capacity. The elution with salt solutions of increasing concentration occurs because the ions in solution compete successfully with the polysaccharide for adsorption on the sites of the ion exchanger.

In a second attempt to fractionate polysaccharide (A)

(Expt.7(5ii)) the DEAE-cellulose was employed in the chloride form and the elution carried out with potassium chloride solutions of increasing concentrations (0·025-1·5 molar), notably with a stronger final electrolyte concentration than in the previous experiment. And this time the total polysaccharide applied to the column was eluted with the neutral medium, giving three distinct peaks (see Graph No.2, p. 36 These fractions however proved to be of nearly identical composition (Table III, p. 36). They contained the same sugars in approximately the same relative proportions, they had similar specific rotations and sulphate contents, and gave identical infra-red spectra. But viscosity measurements indicated that the three fractions had different molecular weights (M.W.) and that M.W. fraction 1 > M.W. fraction 2 > M.W. fraction 3. This is in agreement with the fact that compounds of higher molecular weight are eluted first from an ion exchange column (70).

Fraction 3 from the column and the non-fractionated polysaccharide (A) were examined in the ultracentrifuge. The sedimentation patterns (see Graph No.3, p. 37) indicate that polysaccharide (A) is heterogeneous (i.e. contains molecules of different size), whereas fraction 3 from the column gave a single sharp peak and is apparently homogeneous.

Thus it is seen that none of the experiments carried out to separate the starch-free polysaccharide of U.lactuca into

more than a single component led to any form of chemical fractionation. Until evidence to the contrary is forthcoming polysaccharide A can only be regarded as a single heteropolysaccharide.

Qualitative tests (Expt.8). Polysaccharide (A) was subjected to the routine qualitative tests for the detection of the presence of ester/lactone, keto-sugar, 3,6-anhydro-sugar and amino-sugar. They were all negative except from the Elson-Morgan test for 2-amino-2-deoxy-sugar which was strongly positive. This was of considerable interest, since amino-sugars have never been detected in any seaweed polysaccharide, although staining tests have indicated their presence. Because of the apparently inevitable presence of protein in the green seaweed polysaccharide extracts their acid hydrolysates always contain at least a small proportion of amino acids. And it is known (48) that several amino acids (especially lysine) and even neutral sugars interfere with the Elson-Morgan reaction. The classical procedure of this test was therefore not suitable in the present case for detecting aminosugar. Recently Cessi and Piliego (49) reported a modification of the Elson-Morgan test in which amino acids do not interfere. The principle of the reaction is the same as for the original method, but a distillation procedure is introduced. One utilises the fact that the chromogen, 2-methyl pyrrole (71) formed from a 2-amino-2-deoxy-aldose is volatile and can be easily distilled into the p-dimethylaminobenzaldehyde reagent.

In contrast the chromogens formed from amino acids are non-volatile.

Despite the application of this modified procedure the polysaccharide hydrolysate still gave a positive reaction for aminosugar, although it was not possible to confirm the presence of this sugar by paper chromatography. It was eventually found that the positive aminosugar reaction was due to the presence of sand-hoppers (Amphipoda) which were difficult to discover in the dried weed and remove completely from the crispy, folded fronds of Ulva. The hydrolysates of a cold- and a hot-water extract of a portion of these animals both gave a positive modified Elson-Morgan test and the hydrolysate of the hot water extract on paper chromatography also gave a spot with the same colour and mobility as authentic 2-amino-2-deoxy-D-glucose.

The relative molecular proportions of the sugars present in polysaccharide (A) were estimated by the method of Wilson (50) (Expt.9). This method was found convenient and generally gave a good reproducibility for neutral sugars. No attempt was made to determine glucuronic acid this way, partly because the Wilson method is not particularly suitable for uronic acid estimations and partly because the majority of the glucuronic acid in the polysaccharide hydrolysate probably existed as the rather acid stable 4-O-glucuronosylrhamnose. The molar proportions of glucose:xylose:rhamnose after 6 hours hydrolysis were found to be 1:3.4:4.8, whereas after 16 hours hydrolysis

the proportions were 1:3,5:6,9. The higher proportion of rhamnose obtained on prolonged hydrolysis supports the evidence (28) that a part of the rhamnose in the polysaccharide is joined to glucuronic acid in the more acid resistant uronosyl linkage. Furthermore these results illustrate the necessity of carrying out hydrolyses under identical conditions before comparing the constituents of polysaccharides from different sources.

Desulphation experiments. As mentioned previously the removal of sulphate ester groups is a matter of great importance and delicacy in the study of seaweed polysaccharides but no standard method appears to be available. Grant and Holt (72) reported that the barium salt of diacetone glucose-3-sulphate could be desulphated by the action of lithium aluminium hydride in dioxan solution. Since the acid form of polysaccharide (A) was found to be slightly soluble in dioxan this method was attempted for desulphation (Expt.10(1)), although it was considered that salt formation with consequent decreased solubility in dioxan would occur as soon as the polysaccharide came in contact with the reducing agent. The conditions required for removal of the sulphate groups with lithium aluminium hydride were rather drastic and led to extensive degradation of carbohydrate material. But even the degraded polysaccharide after dialysis and acid hydrolysis proved to contain xylose, glucose, rhamnose, sulphate and trace of uronic acid, thus indicating that all these units must be

present in the inner part of the molecule.

Auto-desulphation of polysaccharide (A) could be carried out very simply by shaking the acid form of the polysaccharide with dry methanol (Expt.10(2)). This led to a significant reduction in the sulphate content (from 19 to 2·4%), but the yield (53%) was not satisfactory, since also in this case some degradation had occurred. This was clearly illustrated by estimation of the relative molecular proportions of the sugars present in the residual polysaccharide, and of the sugars, recovered as methyl glycosides, from the methanol used for the desulphation. The methanol-soluble material contained glucose, xylose and rhamnose in the molar proportions 1:6·3:14·8, while the desulphated, residual polymer, called polysaccharide (B), contained the same sugars in the proportions 1:1·2:1·5. Since in the original polysaccharide (A) the proportions of glucose, xylose and rhamnose were 1:3·4:5, it appears that the desulphation had caused a small loss of glucose, a greater loss of xylose and a still greater loss of rhamnose. The proportions quoted are purely relative, and this makes it difficult to say quantitatively how much of each sugar had been cleaved during the desulphation. An earlier experiment had shown that when the acid polysaccharide was refluxed with methanol for 8 hours considerable degradation took place and only a 10% yield of polysaccharide was recovered. This on hydrolysis and paper chromatography revealed a strong glucose spot together with xylose and rhamnose.

Johnston and Percival (73) were able to remove a proportion of the sulphate groups from carrageenin by the action of methanolic hydrogen chloride, but isolated the desulphated and degraded polysaccharide in only 15% yield. This method of desulphation has proved far more suitable for polysaccharides possessing a proportion of the more acid-stable uronosyl-linkages and chondroitin sulphate was desulphated in this way (51) with very little degradation. McKinnell and E.E.Percival (28) effectively reduced the sulphate content of the E.compressa polysaccharide from 16 to 0·75%, using 0·09M methanolic hydrogen chloride and they recovered the desulphated product in 71% yield. This method, shaking the polysaccharide at room temperature with dry methanolic hydrogen chloride, also proved to be the best for the removal of sulphate from the Ulva polysaccharide (Expt.10(3)), although it was not possible to obtain a product with less than 1% sulphate after a single treatment, even with a stronger concentration of hydrogen chloride. The reason for this might perhaps be that the two batches of polysaccharide (A) used for desulphation contained 18 and 19% sulphate respectively whereas the corresponding Enteromorpha polysaccharide had a sulphate content of only 16%. When polysaccharide (A) was subjected to a repeated treatment with methanolic hydrogen chloride, the sulphate content was reduced to 0·3% with an overall recovery of 50% of polysaccharide, called polysaccharide (C). In a second experiment polysaccharide C' (5% sulphate) was isolated.

Periodate oxidation experiments (Expt.15). Polysaccharide (A) (18% sulphate) and C' (5% sulphate) (Expt.10(3)) were subjected to periodate oxidation initially using the conditions employed by McKinnell and Percival (28) on the closely related polysaccharide of Enteromorpha. These conditions, (0·015 molar $NaIO_4$) under which the sulphated and desulphated Enteromorpha polysaccharide reduced 0·38 and 0·68 moles of periodate per C_6 anhydro-unit respectively, had practically no effect on the corresponding Ulva polysaccharides. The periodate concentration was increased to 0·05 molar (Expt.15), but even then the reduction of the oxidant was only 0·20 and 0·40 moles per C_6 anhydro-unit for polysaccharides (A) and (C') respectively, considerably lower than one would have expected in view of the close resemblance between Ulva and Enteromorpha. This could be connected with the fact, however, that the polysaccharide of E.compressa apparently contained more rhamnose units than that of U.lactuca and had a lower sulphate content (14%) while the Ulva one had 18% sulphate. The recording of the reduction of periodate in terms of "C_6 anhydro-units" is chosen for simplicity. The true consumption of periodate for every sugar residue in a heterogeneous polymer consisting of varying amounts of sulphate, "ash" and protein in addition to the carbohydrate material is obviously very complicated to determine accurately. It must be pointed out also the care necessary both in the interpretation of the results and in the conclusions reached from periodate oxidation experiments on

complex polysaccharide material. Comparative oxidation studies on substances of similar composition are more likely to give valuable information. It is seen in Graph No.4 (p.53) derived from the oxidations that the partially desulphated polysaccharide (C') consumed more periodate than the sulphated material (A). Consequently it may be deduced that removal of sulphate ester groups from the polysaccharide leads to formation of additional α-glycols. It is known that in buffered solution at low temperature vicinal cis hydroxyl groups in sugars are, more or less, selectively oxidised by periodate. It can be seen that at 2° (C') reduces twice as much periodate as (A). This strongly indicates that desulphation furnishes cis rather than trans glycol groupings. The only sugar present in the polysaccharide in which cis glycols occur is rhamnose with cis hydroxyls at C_2 and C_3. Therefore these experiments provide evidence that sulphate groups are linked either to C_2 or C_3 in rhamnose. Evidence that the linkage is to C_2 is derived from infrared examination of the sulphated polysaccharide. A peak at 1240 $cm.^{-1}$ characteristic of the S=O stretching vibration and a second peak at 850 $cm.^{-1}$ corresponding to the C-O-S vibration was given by this material whereas both these peaks were missing from the desulphated polysaccharide. Model experiments on galactose sulphates (86) have shown that the latter peak is characteristic for axial ester sulphate. If this can be applied to all monosaccharides then the sulphate must be attached to C_2 in rhamnose since this

is the only axial hydroxyl group in L-rhamnose in its stable 1C conformation. The same evidence for the existence of 2-sulphated rhamnose units in the polysaccharide of E.compressa (28) has also been obtained. Further confirmation that this is correct has been given by Rees (87) who has studied the rates of acid hydrolysis of various carbohydrate sulphates. These studies showed that the rate of sulphate hydrolysis is dependant upon the type of ester linkage present, sulphates of primary hydroxyl groups being more stable to hydrolysis than those of axial secondary hydroxyl groups which in turn were more stable than those of equatorial secondary hydroxyl groups. The acidic hydrolysis curve for the Enteromorpha polysaccharide showed an initial rapid liberation of sulphate, corresponding to the presence of some equatorial ester, and the rate of sulphate liberation in the latter part of the reaction was consistent with the majority of the sulphate being present as axial ester sulphate. These results not only support the evidence of McKinnell and Percival (28) that rhamnose is sulphated in position 2, but also indicate the presence of some equatorial ester sulphate in keeping with the presence of 2-sulphated xylose (see p. 48). In view of the similarity of Ulva and Enteromorpha it would not be surprising if the water soluble polysaccharide of the latter proved to contain a proportion of sulphated xylose, which can contain equatorially linked ester sulphate only.

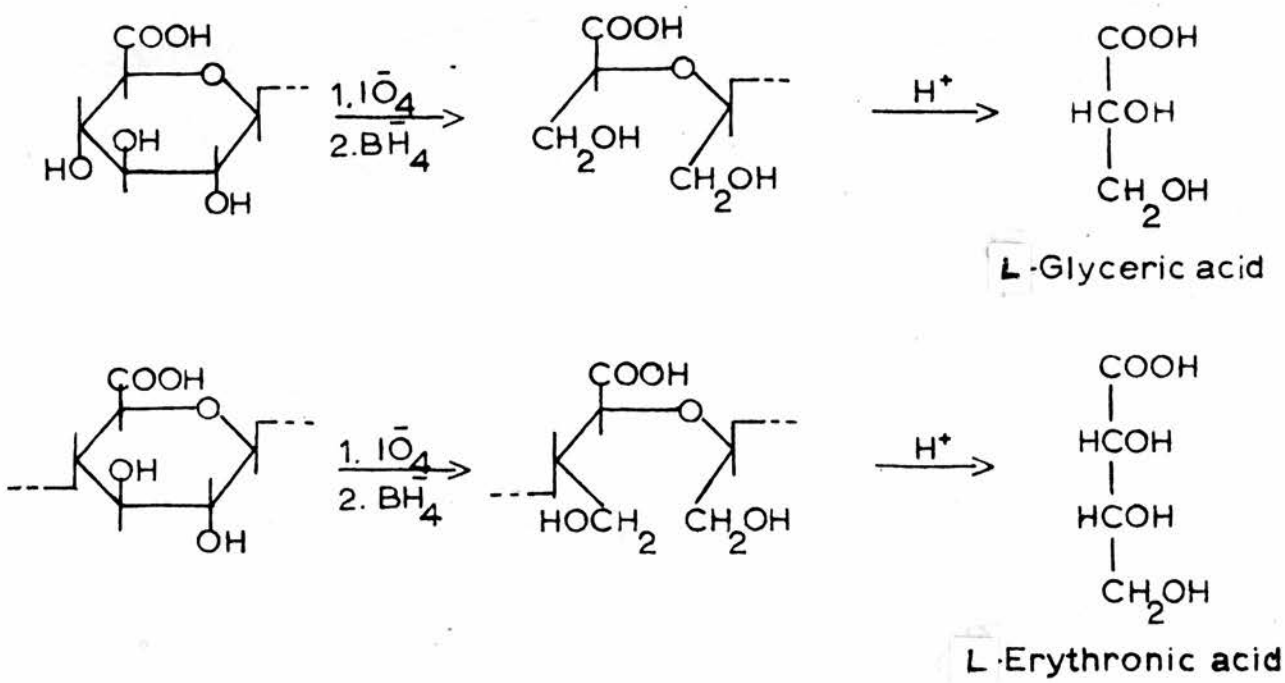
It is to be expected that more periodate is reduced by

both the sulphated and the desulphated polysaccharide at room temperature than at 2° (see Graph No.4, p.53) since a proportion of vicinal trans hydroxyl groups in xylose, glucose and glucuronic acid are undoubtedly cleaved by the periodate, and as mentioned earlier this cleavage occurs more readily at room temperature. However, this difference is only of the order of 20-30% in keeping with the earlier evidence that periodate mainly cleaves the cis glycol groups at C_2 and C_3 in rhamnose, it being remembered that L-rhamnose is the major sugar of this polysaccharide.

Investigation of the oxo derivatives of the sulphated and desulphated polysaccharides (Expt.16). Four different oxo polysaccharides (A_o), (B_o), (C_o) and (C'_o) were prepared from polysaccharides (A) (B) (C) and (C') respectively. They were isolated after exhaustive periodate oxidation at room temperature, by freeze-drying. It is notable that in the absence of buffer no over oxidation seemed to occur and that the reduction of periodate by (A) and (C') was very similar to that in buffered solution (see Table VI and Graph No.4). The greater reduction of periodate by polysaccharides (B) and (C) compared with (C') corresponds to their sulphate content, 2%, 0·3% and 5% respectively. It would appear that the removal of the last 2% of sulphate liberates hydroxyl groups which are adjacent to ones already involved in linkage to other sugar residues and therefore no additional cis glycols are produced.

The relative molecular proportions of the sugars present in the oxo-polysaccharides (Expt.17) are shown in Table VII. As was to be expected the proportions of rhamnose in the desulphated oxo-polysaccharides are considerably smaller, and the proportions of xylose are a little smaller than those of the sulphated oxo-polysaccharide. Paper chromatographic examination of the acid hydrolysates of the oxo-polysaccharides indicated somewhat surprisingly that the desulphated oxo-derivatives still contained a proportion of uronic acid whereas the sulphated oxo-polysaccharide (A_o) apparently was devoid of acid. Consequently the periodate-oxidised and also the non-oxidised polysaccharides were subjected to uronic anhydride estimation (Expt.18). Because of the small amounts of material available the carbazole method (36) was employed. Like any other procedure for the determination of uronic acid the carbazole method is not entirely satisfactory. And in this case it appeared obvious that the brownish colour formed from the neutral carbohydrate material during the period of heating with concentrated sulphuric acid interfered with the reading of the optical density at 520mμ thus giving high results. But even when subtracting an empirically chosen correction factor, obtained from some model experiments on simple sugars, uronic acid-free polysaccharides etc., the desulphated oxo-polysaccharides proved to contain some periodate resistant uronic acid units (see Table VIII). From the uronic anhydride content of polysaccharide (B) and (C') it

appears that the more drastic auto-desulphation, giving product (B), leads to removal of some uronic acid. This is in keeping with the fact that material (B) also contained a low proportion of rhamnose (Table V) indicating that residues of the aldobiouronic acid, 4-O-glucuronosylrhamnose, possibly present as end groups, were probably split off during the desulphation process. It was considered that identification of the cleavage products after periodate oxidation, reduction and hydrolysis would distinguish between uronic acid residues present as end groups and those present in the interior of the polysaccharide chain. According to formulae scheme V a glucuronic acid end group would result in the formation of L-glyceric acid, and a 1,4-linked glucuronic acid residue would give rise to L-erythronic acid:



SCHEME V

The experiments were carried out under controlled conditions in buffered solution and at 2° to avoid over oxidation (60) (Expt.20(1)). The oxidised polysaccharide was reduced with borohydride and the resulting polyalcohol hydrolysed with acid. A number of attempts to locate the expected glyceric and/or erythronic acid by paper chromatography failed, and apparently the only acid spot present was one with the mobility of the unoxidised aldobiouronic acid. A second experiment (Expt.20(2)) carried out in buffer but at room temperature gave the same result although this time the acid spot was very weak, indicating that the glucuronic acid moiety with its trans glycol groupings was more easily oxidised at higher temperature. The complete failure to detect any of the expected cleavage products from the periodate oxidation is difficult to understand.

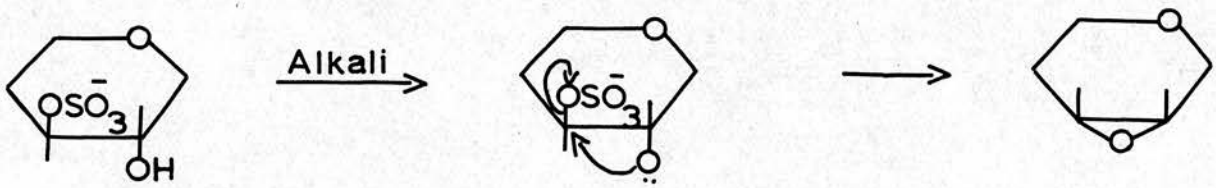
The desulphated polysaccharides (B) (C) and (C') gave a strongly positive ester/lactone reaction (Expt.19(1)) using the ferric chloride-hydroxylamine test (45). That the carboxyl groups had been esterified was to be expected, considering the conditions used for the desulphation process. Kautor and Schubert (51) also reported that during the desulphation of chondroitin sulphate the uronic acid groups were converted into methyl esters. But esterification of the glucuronic acid units of the desulphated Ulva polysaccharide still does not explain the partial resistance to periodate oxidation. It was therefore thought that a proportion of the

esterified glucuronic acid - if present as end groups - possibly had reacted further and lactonised to glucuronolactone residues. This could very well involve a conversion from a pyranose to a furanose ring and give the more stable system of two five-membered rings which would be immune to periodate. Smith (88) showed that glucurone occurs in the furanose form, and Peat et al. (89) found that the action of cold methanolic hydrogen chloride on glucurone led to the formation of methyl glucuronoside while treatment of this with hot acid methanol gave the methyl glycoside of glucopyranosyluronic acid methyl ester. Methanolysis of methylated pectic acid (90) gave a mixture of the pyranose and furanose forms of methyl 2,3-di-O-methyl-galactosiduronic acid methyl ester. Thus it seems apparent that uronic acids under certain circumstances can change from a five- to a six-membered ring and vice versa, even when the glycosidic hydroxyl group is blocked. In view of this it is possible that a part of the uronic acid in the desulphated polysaccharides is resistant to periodate because of lactonisation to glucuronolactone units. Determination of the methoxyl content should normally give information whether the carboxyl groups occurred as methyl ester, lactone, or a mixture of the two. A methoxyl estimation of the desulphated polysaccharides was not carried out however, since it has been shown (117) that it is impossible to remove completely adhering methanol from such materials without degradation. Therefore, to try to distinguish between ester and lactone the respective

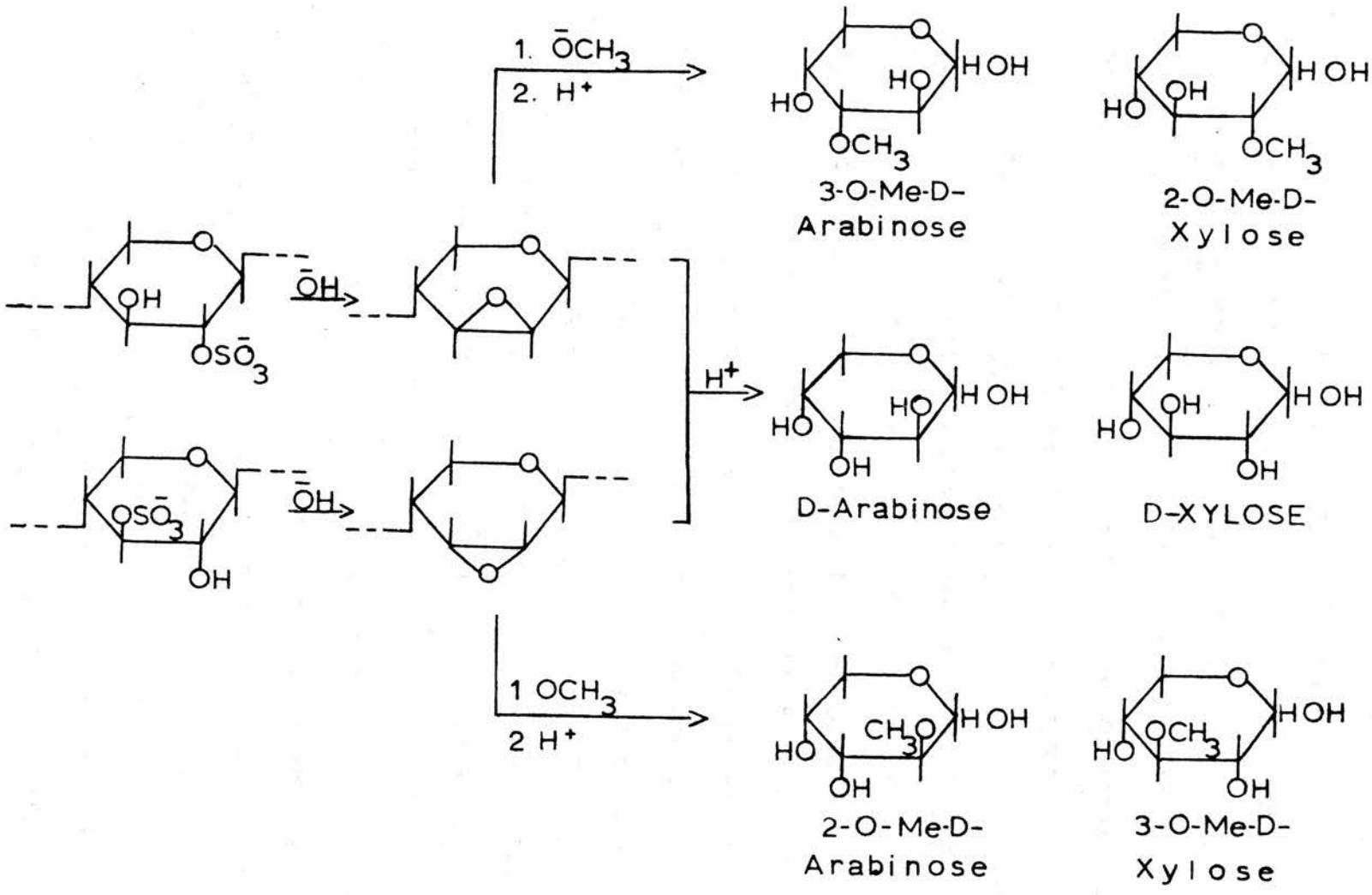
desulphated materials were treated with alkali under very mild conditions (0·1N sodium hydroxide for 3 minutes at room temperature (Expt.19(2)), normally sufficient to open lactones but not cleave ester linkages, before being subjected to the ferric chloride-hydroxylamine test. This resulted rather unexpectedly in a nearly negative colour reaction, indicating that practically all of the uronic acid in the desulphated polysaccharides occurred as lactone. This was apparently inconsistent with the low uronic anhydride content of the desulphated oxopolysaccharides (Table VIII, p. 57). But it must be remembered that a lactone on standing in aqueous solution for several days probably gradually opens and thus becomes vulnerable to periodate. However, this can only be regarded as a tentative explanation of the apparent immunity of the uronic acid to periodate oxidation, and further information is desirable before definite conclusions of the position and linkage of the uronic acid units in this polysaccharide can be drawn.

Desulphation of polysaccharides with alkali rarely leads to satisfactory results. In most cases the carbohydrate material is degraded and little if any of the sulphate removed. But nevertheless the action of alkali on carbohydrate sulphate is of interest because of the diagnostic value this may have for location of the sulphate ester groups on the sugar residues. It has been shown by model experiments on monosaccharide

sulphates (74) that a sulphate ester group having a free trans hydroxyl on an adjacent carbon atom is labile to alkali and will easily form an epoxide ring with a Walden inversion on the carbon atom carrying the sulphate:



In contrast a sulphate ester group situated adjacent to cis-hydroxyls will not be affected by alkali. Opening of an epoxide ring with acid or alkali can occur in two ways and accordingly give two products. The proportion of the products is dependant upon which of the two carbon atoms is more easily attacked by the reagent used. Again a Walden inversion takes place at the carbon atom being attacked. If the reaction is acid-catalysed, the epoxide oxygen is initially protonated but otherwise the process is the same (75). Sulphated polysaccharides behave similarly to monosaccharide sulphates under the action of alkali (74). Consequently on alkali treatment of a sulphated polysaccharide some information concerning the position of the sulphate groups should be obtained irrespective of whether sulphate is removed or not. By treating the Ulva starch-containing sulphated polysaccharide extract with N-sodium hydroxide at 80° in the presence of


1. $\bar{O}CH_3$
2. H^+
3-O-Me-D-
Arabinose
2-O-Me-D-
Xylose
$\bar{O}H$
H^+
D-Arabinose
D-XYLOSE
1 OCH_3
2 H^+
2-O-Me-D-
Arabinose
3-O-Me-D-
Xylose

SCHEME 1

borohydride (52), thus converting reducing end groups into primary alcohol groups, degradation of the carbohydrate was markedly reduced (Expt.10(4)). The polysaccharide material (D) was recovered in 70% yield with the sulphate content reduced from 14·1 to 12·5%. An acid hydrolysate of this product (D) on paper chromatography revealed the presence of two new pentose sugars, the faster one only as a trace, in addition to the glucose, xylose, rhamnose and mannose present in the original extract. Paper chromatographic examination in various solvents tentatively identified the two pentoses as arabinose and lyxose (trace). Both sugars were subsequently separated on thick paper. The arabinose crystallised and was characterised as D-arabinose by melting point, rotation and the formation of the benzoylhydrazone. The syrupy lyxose was chromatographically and ionophoretically pure and was tentatively identified as D-lyxose by its negative rotation.

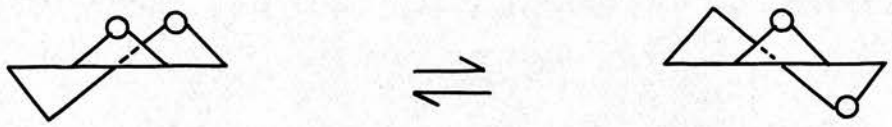
The formation of arabinose can be simply explained if the polysaccharide contains 1,4-linked xylose residues carrying sulphate and a free hydroxyl in positions 2 and 3 (see formulae Scheme I, p. 84). As is seen from the formulae mainly β-linkages are thought to be present in view of the negative rotation of the polysaccharide. The arabinose can only have arisen from xylose since neither glucose, rhamnose nor glucuronic acid could possibly have given this sugar under the conditions of the experiment. Estimation of the relative molar proportions of the sugars in polysaccharide (D) (50)

(Expt.12) showed that it contained glucose (1·0) xylose (1·6) rhamnose (2·0) and arabinose (0·1). By correcting for the content of ash, protein, sulphate and uronic acid it was possible to estimate that 1·1% of arabinose was formed. The decrease in sulphate content, 1·6%, corresponds to 1·6 x 150/96 = 2·5% pentose of which 1·1% is arabinose and consequently 1·4% is xylose. This means that opening of the epoxide ring yields 1·1 x 100/2·5 = 44% arabinose and 1·4 100/2·5 = 56% xylose, provided that the sulphated rhamnose is not affected during the alkali treatment. The quantity of lyxose formed was so small that it scarcely affects the above calculations. The removal of ca. a tenth of the sulphate by alkali corresponds to monosulphation of about 15% of the xylose units.

The arabinose on isolation proved to be the expected D-arabinose. But the formation of this sugar was not sufficient to distinguish between C_2- and C_3-sulphated xylose units. However cleavage of an epoxide ring with sodium methoxide rather than acid results in the formation of methyl derivatives, the methoxyl groups entering the sugar units at the site of attack. It follows that xylose-2-sulphate would yield 2-O-methyl xylose and 3-O-methylarabinose whereas in the products from 3-sulphated xylose the position of the methyl groups would be reversed (see formulae Scheme I, p. 84). This method of opening the epoxide rings in polysaccharide (D) was investigated in spite of two possible complications: Firstly, the polysaccharide, which was insoluble in methanol,

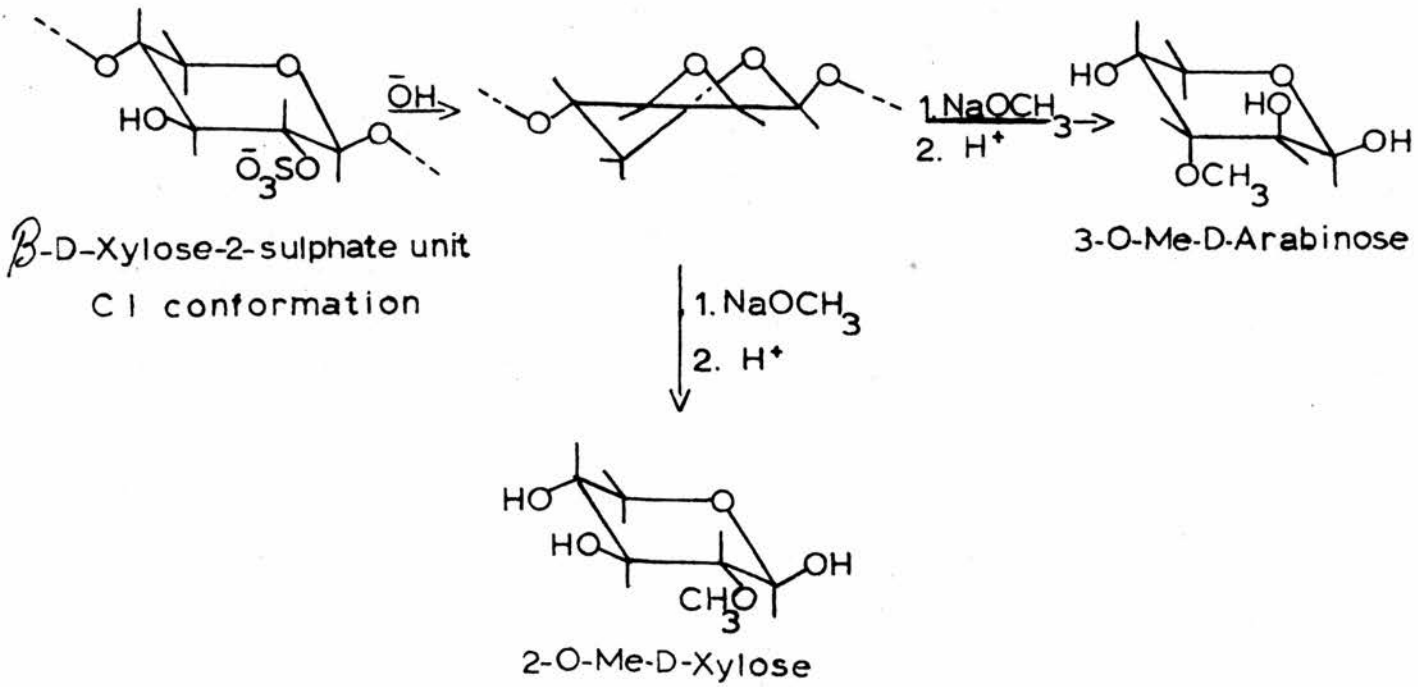
would have to react in the solid state. Secondly, sulphated polysaccharides are extremely difficult to completely free from absorbed moisture; they tend to retain some water even after the most intensive drying. The presence of water might prevent the formation of any methylated sugars and only lead to the production of arabinose and xylose as before. In spite of these difficulties it proved possible to introduce a methyl group by refluxing polysaccharide (D) with N-sodium methoxide in dry methanol (Expt.13). The product on paper chromatography after acid hydrolysis gave a spot corresponding to 2-O-methylxylose and several trace spots, possibly of other methylated pentoses in addition to unmethylated glucose, xylose, rhamnose, mannose and arabinose. No spot with the mobility of the expected 3-O-methylarabinose could be detected. The only methyl sugar formed in significant quantity was isolated and characterised as 2-O-methylxylose, thus proving that xylose is sulphated in position 2. However, it is difficult to explain the apparent complete absence of 3-O-methylarabinose. As mentioned previously the anhydro rings of sugar epoxides invariably open with inversion to give trans products and also in most cases to give diaxial products. It has been suggested (76) that Fürst and Plattner's rule (77) of diaxial opening of steroid epoxides may also be applied to a number of sugar epoxides, in particular those with their conformation stabilised by a 1,6-anhydro ring or a 4,6-benzylidene group. But also with any other sugar epoxide it will seem obvious that diaxial

ring-opening involving the coplanar transition state favourable for a S_N2 reaction, should generally be preferable to di-equatorial opening which is a much more hindered process (78). In view of this the 2-sulphated xylose residues in the polysaccharide, if occurring in the more stable $^4C_1$ conformation (79), on epoxide ring formation and subsequent opening with a nucleophilic reagent would be expected to give predominantly the arabinose derivative (see formula scheme II, p. 88).. Monocyclic sugar epoxides however have a flexible conformation and can exist in two "half chair" forms (80):

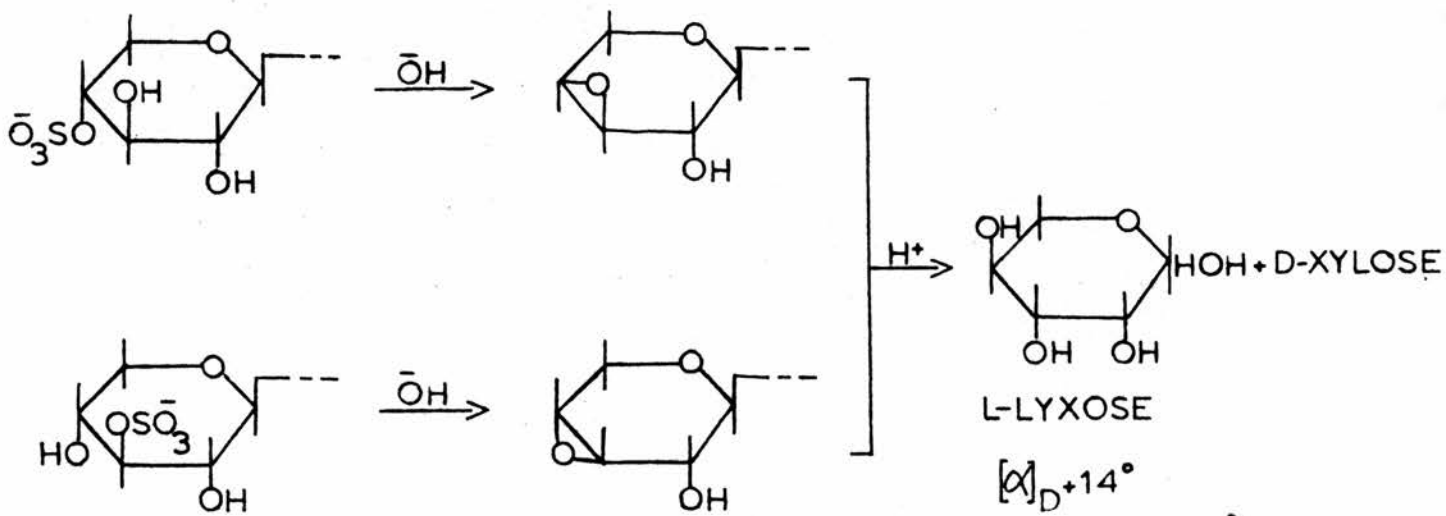


I ⇌ II

If it is accepted that diaxial opening is the rule and that the direction of ionic ring-opening is dependent on the conformation of the epoxide derivatives (81) it should be unnecessary to postulate exceptions to Fürst and Plattners rule. On these lines it would be possible to understand the formation of the "diequatorial product" 2-O-methylxylose. Thus if the sugar epoxide units in the present polysaccharide molecule are already held in the conformation II, or if they are flexible enough to change into this conformation, then 2-O-methylxylose would be produced by diaxial opening and this would readily change back



SCHEME 2

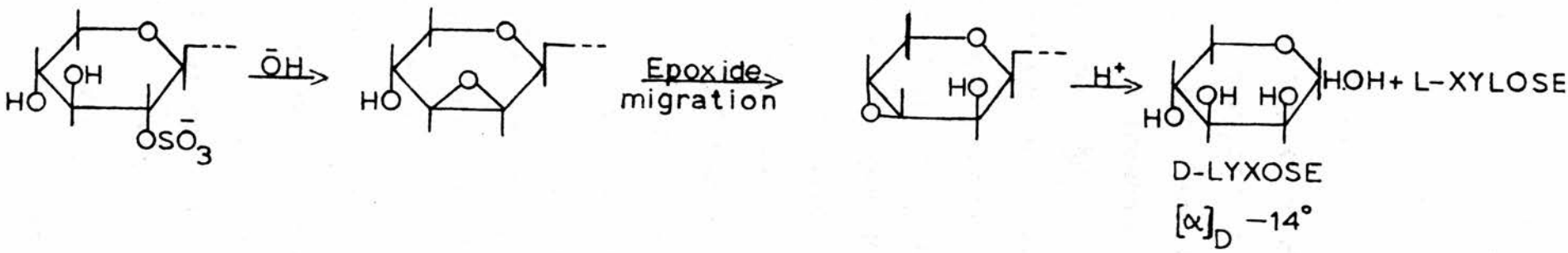


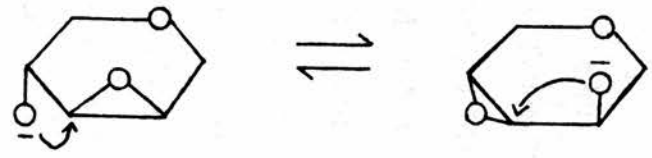
SCHEME 3

into its more stable Cl conformation. However, in the writer's opinion these sugar epoxide residues in a polysaccharide molecule are too rigid to change their conformation and it is regarded more probable that steric as well as other effects play an important part in both the formation and opening of these anhydro rings. It is noteworthy that Aspinall and Ross (82) on cleavage of a pentose disaccharide 2,3-epoxide with sodium methoxide obtained the methylated xylose derivative only, again an apparent diequatorial opening.

As mentioned previously the hydrolysate of polysaccharide (D) after being refluxed with sodium methoxide contained a significant proportion of arabinose. This could be due to a) the presence of some residual water in the polysaccharide, b) a proportion of the anhydrorings remaining uncleaved until the final acid hydrolysis of the product or c) that a proportion of the anhydrorings had been opened during the initial treatment with N aqueous alkali. The last is the most likely explanation and consequently the yield of monomethyl sugar might have been better if the sulphated polysaccharide had been treated directly with sodium methoxide. Unfortunately lack of time and material prevented confirmation of this.

The simplest explanation for the formation of lyxose, which was detected in trace quantity in the hydrolysate of polysaccharide (D) is that it arose from xylose end groups sulphated on C_3 or C_4. Either of these would give L-lyxose after alkali treatment and hydrolysis, according to formulae


HO
OH
OSO3
ŌH
HO
Epoxide
migration
HO
H+
HO
OH HO
HOH+ L-XYLOSE
D-LYXOSE
[α]D −14°


Ō

SCHEME 4

scheme III (p. 88). But since the derived lyxose had a negative rotation, it must be the D- and not the L-form of this sugar, and consequently it could not have arisen by this route. It would be more obvious, perhaps, to expect the xylose end groups to carry sulphate on C_2, in the same way as the rest of the sulphated xylose units. And if this is the case the formation of lyxose can be explained by epoxide migration. Experiments on model sugar sulphates (83) have shown that epoxide ring migration occurs under alkaline conditions if there is a free trans hydroxyl group adjacent to the epoxide ring. The trans hydroxyl anion acts as a nucleophilic agent and attacks from the opposite side to the anhydroring oxygen with Walden inversion and the formation of a new anhydroring. Any 2-sulphated xylose present as an end group could give rise to D-lyxose by this mechanism, (see formula scheme IV, p. 89). Examples of epoxide migration have been encountered in the polysaccharide as well as in the monosaccharide field. Ricketts and Overend (84) (85) observed the migration of a 2,3-epoxide to the 3,4-epoxide during the alkaline desulphation of a dextran sulphate.

Further evidence that the lyxose did not arise from 3-sulphated xylose end groups was obtained from the fact that a hydrolysate of the polysaccharide which had been oxidised by periodate before alkali treatment was devoid of lyxose (Expt.14). 3-Sulphated xylose units would be immune to periodate oxidation and should therefore yield lyxose on

alkali treatment after oxidation. At the same time it is worth noting that a smaller quantity of arabinose was obtained from the periodate oxidised, alkali treated polysaccharide. This is in keeping with the fact that some, but not all of the xylose-2-sulphate is present as end groups. From the above evidence it follows that 2-sulphated xylose units are present in the polysaccharide of U.lactuca. This is the first time that a sulphated pentose has been found in nature and also the first time that direct evidence has been obtained for the site of a sulphate group in a green seaweed polysaccharide.

Conclusion.

The present work fully supports earlier findings that the water-soluble polysaccharides of U.lactuca, E.compressa and A.centralis resemble each other closely, indicating that these algae have a very similar carbohydrate metabolism and constitute a distinct group within the green seaweeds. Like other green algae, each of these weeds metabolises a small proportion of a starch. Apart from the separation of this glucose polymer the water-soluble extracts of U.lactuca, E.compressa and A.centralis have persistently resisted fractionation into different polysaccharides, thus indicating that the major water-soluble material constitutes a single heteropolysaccharide. This contains glucose, xylose and rhamnose as the main constituent sugars in addition to sulphate ester groups and glucuronic acid residues. The aldobiouronic acid,

4-O-glucuronosylrhamnose, never previously found in nature, has been shown to be present in these three seaweeds, possibly as end groups. The sulphate groups of Enteromorpha and Ulva are mainly linked to C_2 of rhamnose, but Ulva also contains a portion of 2-sulphated xylose, of which some is present as end groups and some occurs as 1,4-linked xylose-2-sulphate residues. The Ulva polysaccharide reduces a smaller amount of periodate than Enteromorpha and Acrosiphonia, and even the desulphated material from Ulva consumed only ca.0·5 mole periodate for every C_6 anhydro-unit, indicating a high proportion of 1,3-linkages. Evidence has been obtained that the glucose units are mainly situated in the inner part of the molecule, since the polysaccharide after partial degradation contains a relatively higher proportion of glucose. Now that it has been found possible to desulphate this polysaccharide it is hoped that partial hydrolysis and methylation studies, in correlation with the results of the present work, will reveal much of the fine structure of this polysaccharide.

PART TWO

A CONTRIBUTION TO THE STRUCTURE OF ALGINIC ACID.

INTRODUCTION.

Alginic acid constitutes the principal structural carbohydrate of the brown seaweeds, where it occurs together with other polysaccharides such as fucoidin and laminarin. This polyuronic acid was discovered by Stanford (91) in 1883 and has since become a product of great commercial importance. This is primarily due to the hydrophilic colloid properties of the alginates and their ability, at low concentrations (ca. 0·1-2%), to form stable viscous solutions or gels. Since alginic acid is harmless to eat, alginates, particularly the sodium and calcium salts have found extensive use in the food industry and also in the production of pharmaceuticals and cosmetics wherever an emulsifying or thickening agent is required. The vast field for the application of alginates has been reviewed by McDowell (92).

All the brown seaweeds contain a considerable amount of alginic acid but the content varies within a range of ca.10-30% from species to species and from season to season, especially in the fronds (93). Alginic acid itself is insoluble in water and is usually extracted as the sodium salt with dilute aqueous sodium carbonate, giving highly viscous solutions. The viscosity of these solutions varies greatly from sample to sample and the differences are thought to be due to the varying chain length of the polysaccharide. The sodium alginate solution on acidification gives the free acid as a gelatinous precipitate. Similarly addition of aqueous calcium chloride

to a sodium alginate solution precipitates the insoluble calcium alginate.

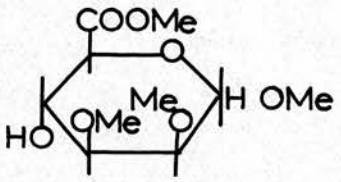
Although alginic acid was one of the earliest of the algal polysaccharides to be studied in detail, the progress in establishing its molecular structure has been very slow. This must be mainly attributed to its marked resistance to hydrolysis under conditions that do not destroy the products. Stanford considered alginic acid to be a weak, nitrogen-containing organic acid and prepared a variety of different salts with metals as well as with organic bases (94). Later investigations of the pure acid however revealed that it was free of nitrogen (95)(96). Evidence that alginic acid was of a polysaccharide nature was obtained by Kylin (97) who reported the presence of pentose sugar in the acid hydrolysate. Atsuki and Tomada (98) suggested that uronic acid was present as they found that 19.9% of carbon dioxide was produced on boiling alginic acid with 12% hydrochloric acid, a standard reaction for uronic acids. Schmidt and Vocke (99) claimed to have obtained D-glucuronic acid on hydrolysis of alginic acid and isolated it as the cinchonine salt. But this was inconclusively identified by its melting point only. The investigations of Nelson and Cretcher (96)(100)(104) advanced the knowledge of the chemistry of alginic acid considerably. They obtained from their pure preparations of the acid a neutralisation equivalent of 176-184, and decarboxylation with 19% hydrochloric acid gave carbon dioxide (24·7%) corresponding

to a uronic acid content of 100%. Carbon and hydrogen analysis indicated the formula $(C_6H_8O_6)_n$; furthermore alginic acid gave a negative Fehling's test, but a strongly positive naphthoresorcinol uronic acid test, and it was concluded that this polysaccharide was a polyuronic acid with all the carboxyl groups free and all the aldehydic groups involved in linkage. The C_6 aldehydic acid obtained by hydrolysis under more drastic conditions than used previously, was shown to be neither glucuronic nor galacturonic acid. The identity of this uronic acid was indicated by oxidation to the dibasic acid whose diamide and diphenylhydrazide had the properties of the corresponding derivatives of authentic D-mannosaccharic dilactone. Confirmation of this was obtained when D-mannuronic acid itself, as the lactone, was isolated from hydrolysates of Macrocytis pyrifera (100) . The findings of Nelson and Cretcher have since been confirmed by other workers (101) (102)(103).

Alginic acid on treatment with 10% methanolic hydrogen chloride under anhydrous conditions yielded a water-soluble degraded polymer of considerably lower viscosity than the original material (105). The supernatant solution contained the methyl ester of methyl D-mannuronoside which after methylation, hydrolysis and oxidation furnished 2,3,4-tri-O-methyl-D-mannosaccharic acid. The residual degraded polysaccharide after methylation proved exceptionally stable towards hydrolytic agents. However when boiled with 50% nitric acid

it underwent hydrolysis followed by degradative oxidation with the formation of di-O-methylerythraric acid (I). This indicated that in each of the mannuronic acid residues the methyl groups were attached either to C_2 and C_3 or to C_4 and C_5.

I



II

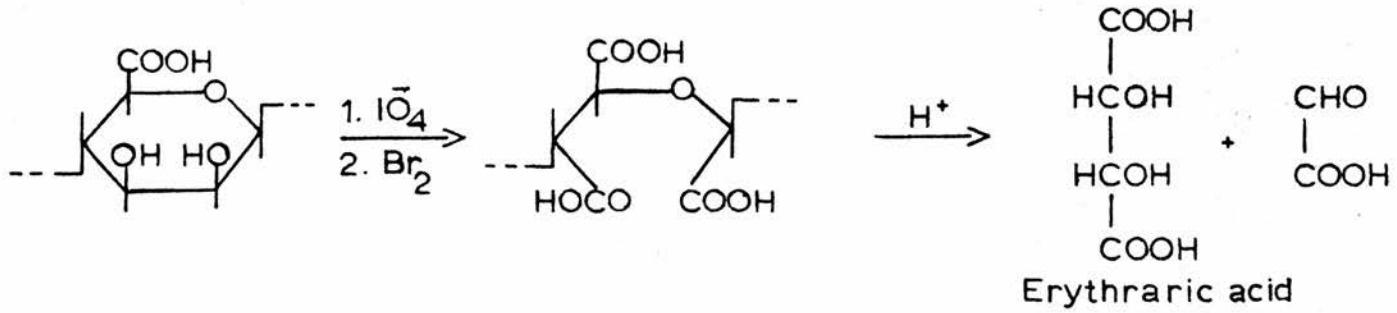
Proof that they were situated at C_2 and C_3 was provided by heating methylated alginic acid under pressure with 4% methanolic hydrogen chloride at 150° to give the methyl ester of methyl 2,3-dimethyl-D-mannuronoside (II). As this compound on hydrolysis to 2,3-di-O-methyl-D-mannuronic acid underwent a change in rotation it is evident that an oxide ring was present and that substitution on C_4 and C_5 is precluded (105). It would appear therefore that the D-mannuronic acid units in alginic acid are linked either 1,4 (pyranose ring structure) or 1,5 (furanose ring structure). The extreme stability of alginic acid makes a pyranose structure highly probable, and considering the large negative rotation ($[\alpha]_D$-139°) it would appear that mainly β-linkages are present. These results were later confirmed (106) by structural studies on a less degraded alginic acid with a chain length of ca.100 units.

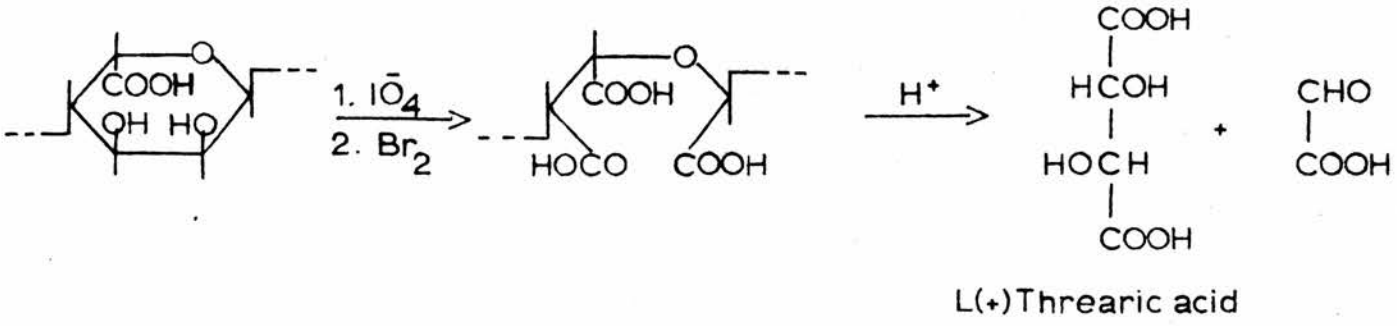
Methylation of this acid and subsequent formic acid hydrolysis was followed by esterification and glycosidation of the products. These on reduction with lithium aluminium hydride and acid hydrolysis resulted in the formation of 2,3-di-O-methylmannose as the major component with smaller amounts of 2,3,4-tri-O-methylmannose, monomethylmannose and dimethylglucose. This "glucose" derivative was shown by model experiments, not to arise from C_2-epimerisation of 2,3-di-O-methylmannose under the alkaline conditions of the reduction. As the dimethyl "glucose" was not fully characterised, it might in view of more recent investigations (107), perhaps have been a methylated gulose.

X-Ray diffraction studies carried out on fibres of alginic acid and of sodium alginate have resulted in well-defined diffraction patterns, indicating a high degree of orientation (108). In this respect alginic acid resembles cellulose and pectic acid. It has been shown, however, that the projection per pyranose unit along the fibre axis of alginic acid is 4·37Å, while that of cellulose is 5,13Å and that of pectic acid 4·30Å (109). This is because the angle between the rings in alginic and pectic acid is larger and the molecules therefore more buckled. Sodium alginate, however, has a projection unit of 5·0Å while sodium pectate has one of 4·37Å (109). It has been pointed out (110) that this difference between alginic acid and sodium alginate corresponds to the existence of the acid units in the former in the 1C conformation and in

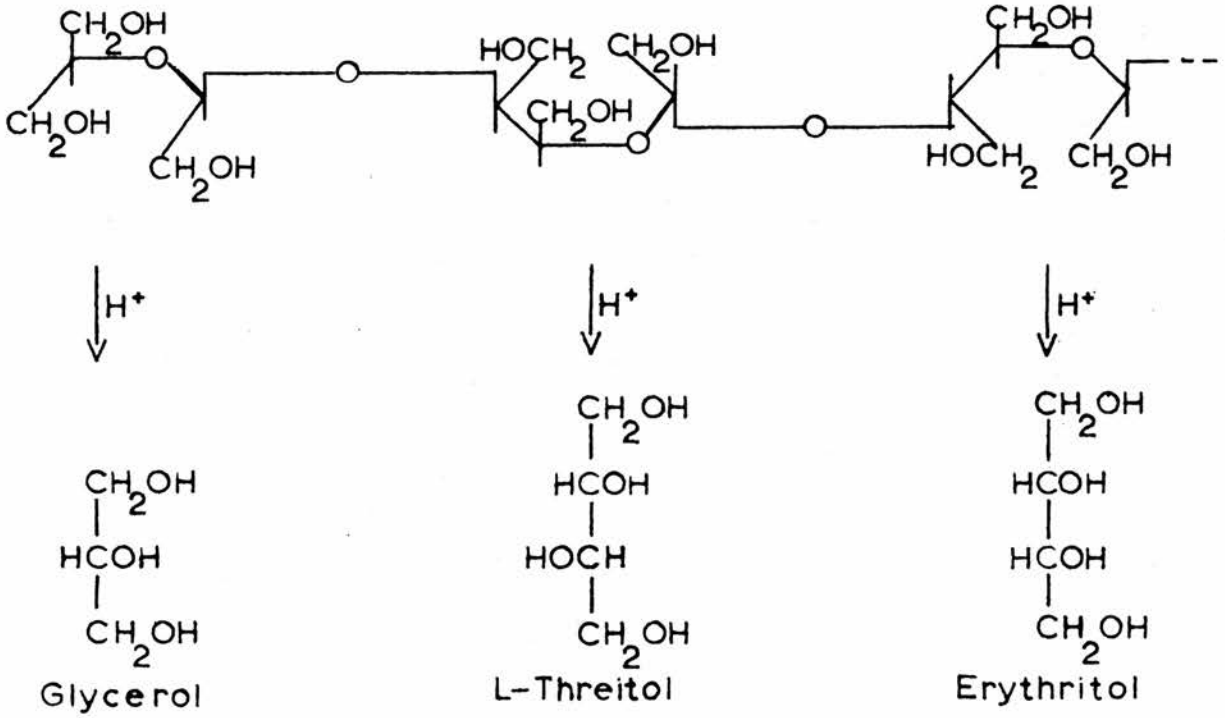
the latter in the Cl conformation. A mannuronic acid unit in the 1C conformation will have its glycosidic β hydroxyl as well as its C_4 hydroxyl in axial positions. Accordingly, since alginic acid is β 1,4-linked an angle of ca.90° will exist between the pyranose rings in the chain. On the other hand in cellulose and sodium alginate, assumed to occur in the Cl conformation, the β glycosidic and C_4 hydroxyl groups are in equatorial positions, thus giving an angle of ca.20° between the rings in the chain. If these considerations can be applied to pectic acid and its sodium salt it would follow that they both occur in the Cl conformation. Pectic acid, essentially regarded as an α-1,4-linked polygalacturonic acid, will with its units in the Cl conformation have both the α-glycosidic and the C_4 hydroxyl in an axial position, thus making the angle between the rings ca.90°.

Until recently available evidence indicated a straight chain of 1,4-linked β-D-mannuronic acid residues as the structure of alginic acid. However, with improved chromatographic techniques Fischer and Dorfel (107) showed that L-guluronic acid in addition to D-mannuronic acid was present in alginic acid from twenty-two species of brown algae. It should be emphasised at this point that all the earlier work on alginic acid had failed to account for more than 45% of the original molecule (111). This together with the acid lability of guluronic acid explains the failure of previous investigators to isolate guluronic acid. That guluronic acid in fact is





SCHEME 6



SCHEME 7

present in the polysaccharide has been confirmed by other workers (112)(113). In order to ensure that this acid was not an artefact of acidic hydrolysis and also to obtain evidence for the mode of linkage in the polysaccharide, periodate oxidation followed by bromine oxidation and hydrolysis was carried out. If alginic acid consisted entirely of 1,4-linked mannuronic acid residues, then this procedure would furnish a mixture of glyoxylic and erythraric acids with zero rotation. The presence of 1,4-linked guluronic acid residues, however, would, in addition, give rise to L(+)threaric acid, and the final mixture would have a positive rotation, which was indeed shown to be the case (112) (Formulae Scheme VI).

Periodate oxidation experiments on alginic acid, under controlled conditions (60)(114) revealed that ca.0·5 mole of periodate was reduced for every C_6 anhydro-unit which is less than would be expected from a chain of 1,4-linked hexuronic acid residues. The free aldehydic groups of the oxo-alginic acid were reduced with borohydride to primary alcoholic groupings, as were the carboxyl groups after esterification with diazomethane. Chromatographic analysis of the hydrolysate revealed the presence of mannose, gulose, erythritol, and glycerol (110). The mannose and gulose could only have arisen from unoxidised mannuronic and guluronic acid units in the alginic acid. Erythritol and threitol are derived from oxidised mannuronic and guluronic acid residues respectively, similarly cleaved between C_2 and C_3. Glycerol is produced

from the non-reducing end groups of the polymer chains (Formulae Scheme VII). This mixture on separation on thick paper yielded glycerol (1 part), mannose + gulose (8 parts), erythritol + threitol (11 parts). Since molecular-weight determinations of alginic acid gave a minimum value of 200 anhydro-uronic acid units, it is difficult to understand the high proportion of glycerol separated from the hydrolysate. However, Goldstein et al. (115) were likewise unable to account for glycerol in a hydrolysate from similarly treated cellulose.

The low reduction of periodate by alginic acid is surprising since all the evidence indicates that the residues are 1,4-linked and therefore vulnerable at C_2-C_3. Kaye and Kent (116) obtained a positive colour reaction for ester/lactone (the ferric chloride-hydroxylamine test) and concluded after quantitative estimations that in dry alginic acid the acid residues existed chiefly as the lactone. In order to form a 3,6-lactone the mannuronic acid units must assume the unstable 1C conformation with the C_3 hydroxyl and the C_6 carboxyl in an axial position. In view of the X-ray studies (108)(109), indicating that the pyranose rings in alginic acid occur in the 1C conformation it appears that lactone formation would be possible, and the 3,6-lactone groups, if formed, would be expected to have a stabilising effect on the rings in their more labile conformation. The presence of lactone groups in the polysaccharide would explain the partial

immunity of the mannuronic acid residues to periodate oxidation under the conditions of the experiment. However, uncleaved guluronic acid is also found after periodate oxidation, and this hexuronic acid cannot form a 3,6-lactone since the carboxyl and the C_3-hydroxyl groups are trans to one another. On the other hand guluronic acid in its furanose form could easily form a 3,6-lactone. It must however be pointed out that Kaye and Kent (116) would have obtained the same positive colour reaction in the case of intermolecular ester linkages. The presence of ester linkings between adjacent chains, the carboxyl group of one chain being linked to the hydroxyl group of C_2 or C_3 of another would also explain the immunity to periodate oxidation, both for the mannuronic and guluronic acid units. The infrared spectrum of alginic acid in Nujol mull in the carbonyl region in fact favours the presence of ester with a peak at 1745-1750 $cm.^{-1}$, rather than lactone linkages.

The reason for the small periodate uptake by alginic acid might also be the presence of 1,3-linkages in the chain. No evidence for such linkages has previously been obtained, but in view of the inability of any group of workers to obtain theoretical yields from this polysaccharide the possibility cannot be precluded.

PART TWO of this thesis mainly describes epimerisation experiments on uronic acids, reduction of alginic acid to a nearly neutral polysaccharide and attempted partial hydrolysis

of this material. A significant part of this section has already been published (see J.Chem.Soc., 1962, pp.1213-1214).

EXPERIMENTAL

Expt.1. The effect of Heat on Aqueous Solutions of Uronic Acids at Different pH.

(a) At pH 4·5 0·01M-solutions of D-mannurone and D-glucurone (200 ml. each) in distilled water were refluxed during 24 hours. The solutions slowly became brown and dark-coloured precipitates were deposited. At intervals the pH of aliquots (2 ml.) were determined.

TABLE I.

Period of heating (hr.)	0	3	8	24
Mannurone solution pH	4.30	3·10	2·95	2·90
Glucurone solution pH	5·15	3·10	2·90	2·85

After 24 hours a portion of each solution (50 ml.) was withdrawn and filtered, and the filtrate was neutralised to pH 6 with sodium hydroxide solution and then freeze-dried to a cream coloured solid (50 mg. ca.50%).

(b) At pH 7·0. A further 50 ml. were withdrawn, filtered and the filtrate after adjustment to pH 7 with sodium hydroxide solution was reheated at 100° for 2 hours. The resulting solutions were decolourised with charcoal and concentrated to dryness. The four solids obtained, after conversion to the free acid with Amberlite IR-120(H^+) resin were examined chromatographically in solvents (A),(B) and (H). In each case the main spot was that of the original acid;

a faint spot corresponding to the lactone could be observed in the chromatogram of the solid derived from mannurone which had been heated for 24 hours in distilled water. In addition a spot $R_{glucuronic\ acid}$ 1·47 (solvent B) could be detected from both the mannurone and glucurone on paper sprayed with aniline oxalate or bromocresol green, when an increased quantity of substance was spotted on the chromatogram. No spots corresponding to (a) guluronic acid or its lactone could be detected in the materials derived from mannuronolactone or to (b) a second substance from glucuronolactone.

About half of each solid (dried overnight in a vacuum desiccator) was refluxed with 4% methanolic hydrogen chloride (2 ml.) for 7 hours. After neutralisation with silver carbonate the derived syrup was dissolved in water (2 ml.) and the solution treated with potassium borohydride (50 mg.). The mixture was set aside for 20 hours, then neutralised with acetic acid and finally hydrolysed with N-sulphuric acid at 100° for 3 hours. After neutralisation with barium carbonate, filtration, deionisation and concentration the products were examined chromatographically in solvent (E) and ionophoretically in borate buffer at pH 10. The two products from mannurone gave a single spot identical with mannose run as control, and those derived from glucurone gave a single spot corresponding to glucose.

Expt.2. Subjection of Mannurone, Methyl Methyl Mannosiduronate and Glucurone to Conditions Used in the Extraction of Alginic Acid from L.digitata.

D-Mannuronolactone (0·25 g.), methyl methyl mannosiduronate (0·1 g.) and D-glucuronolactone (0·25 g.) each in water (100 ml.) were stirred with an equal weight of calcium hydroxide under nitrogen for 30 minutes at 60°. Sulphuric acid was then added until the solutions were 0·2N with respect to acid, and the mixture left for 1 hour with occasional shaking. Neutralisation was then effected with sodium carbonate and excess sodium carbonate added to give an approximately 3% solution. The mixtures were kept at 50-60° for 3 hours under nitrogen and then brought to pH 6 with sulphuric acid and concentrated to about 50 ml. After partial neutralisation with barium carbonate and filtration, the filtrate was freed from sulphate ions by the addition of barium chloride. Deionisation was then achieved with IR 120(H) resin, silver carbonate, hydrogen sulphide and finally with charcoal: The respective solutions were applied to a column containing Ultrasorb S.C.120/240 charcoal (118) (30 g.) which was then washed with water (200 ml.) to remove ions, and the carbohydrate materials subsequently eluted with 25% ethanol (200 ml.). The solution containing the methyl ester methyl glycoside after concentration to a syrup was hydrolysed for 5 hours at 100° with N-sulphuric acid, and then neutralised, deionised and concentrated again. The three

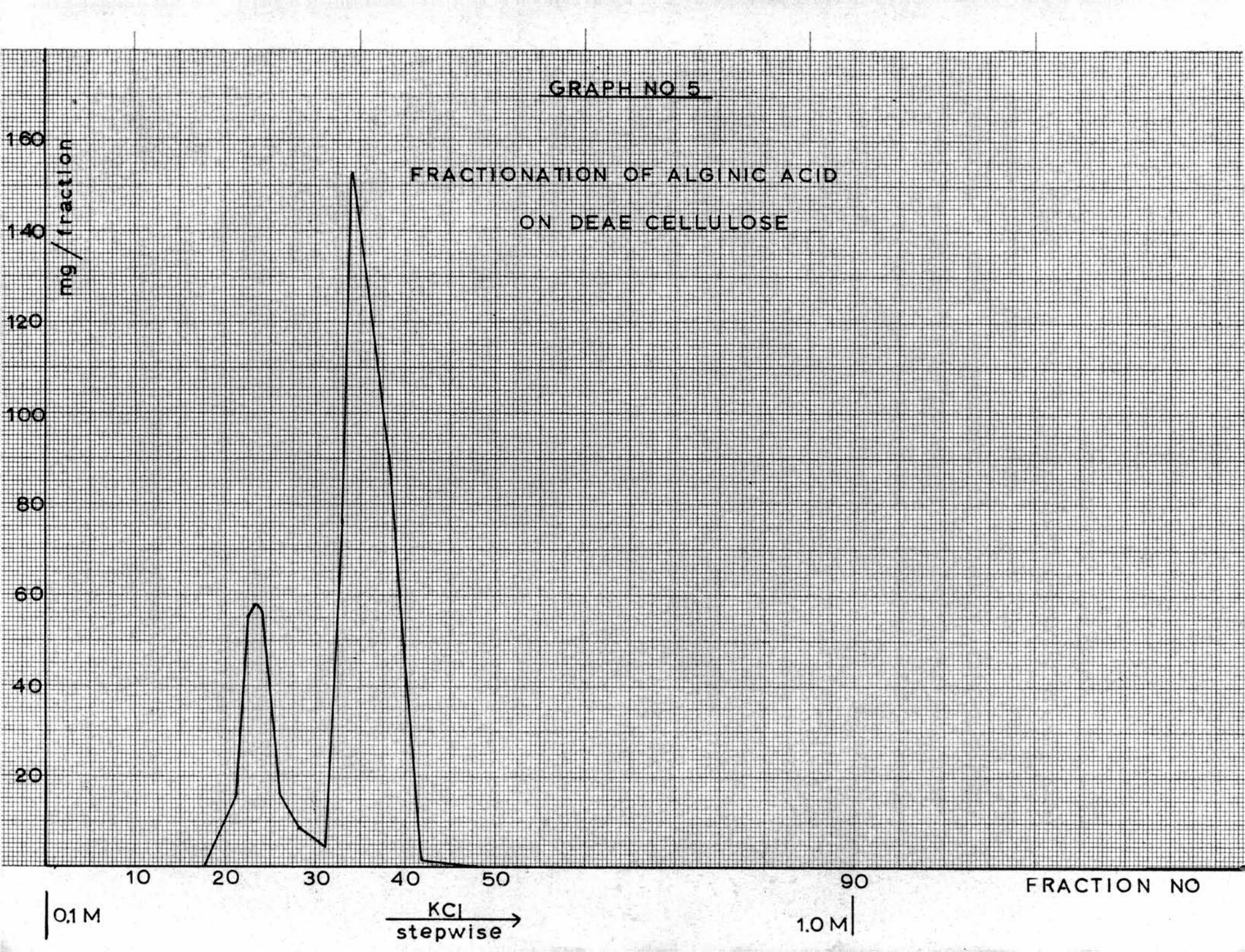
derived materials were subjected to chromatographic analysis in solvent (H) for 60 hours. The syrup derived from glucurone showed the presence of two spots, the one corresponding to glucuronic acid and the other with $R_{Glucuronic\ acid}$ 1·26 (probably L-iduronic acid). That derived from mannurone showed the presence of three spots, of which two corresponded to D-mannuronic and L-guluronic and/or D-glucuronic acids. The third spot which had $R_{Glucuronic\ acid}$ 1·88 was not identified. In contrast the syrup derived from the ester glycoside gave only one spot corresponding to mannuronic acid. Chromatograms eluted for 6-8 hours in solvent (G) also revealed spots corresponding to the respective lactones in each of the materials.

Expt.3. Detection of L-Guluronic Acid after Direct Acid Hydrolysis of the Seaweed Laminaria digitata.

(1) With sulphuric acid (107). The dried, ground fronds (ca. 0·5 g.) were treated with 80% sulphuric acid (2 ml.) at 20° for 15 hours. After dilution with water (20 ml.) to 1·6 N-sulphuric acid, the mixture was heated in a stoppered tube for 6 hours at 100°. Neutralisation with the calculated amount of calcium carbonate and filtration gave a dark brown filtrate which was decolourised with charcoal. Deionisation and concentration gave a syrup. Lactonisation of a portion of this syrup was attempted by heating with 0·25 N-hydrochloric acid for 2 hours at 100° (107). This solution as well as the

syrup, when subjected to paper chromatography, revealed spots corresponding to mannuronic and guluronic acid (solvent H, 60 hours) and to mannurone and gulurone (solvent G, 6-8 hours). Glucose and fucose could also be detected.

(2) With formic acid. The dried, ground fronds (ca.1 g.) were mixed with 0·2N-sulphuric acid (20 ml.) and shaken vigorously for some minutes and then left overnight. After filtration the powdered seaweed was heated under reflux with 75% formic acid (30 ml.) in a boiling water bath for 12 hours. Undissolved material was removed by filtration and the filtrate was distilled in vacuo to remove most of the formic acid, the last traces of which were removed by repeated distillation with water. The residue was dissolved in water, treated with charcoal, filtered and concentrated to a syrup which was hydrolysed with N-sulphuric acid (5 ml.) for 3 hours at 100° to destroy any stable formyl esters. Sulphate was subsequently removed by shaking the solution with 5% (v/v) N-methyldioctylamine (119) in chloroform solution. Residual traces of amine were removed with 1R-120(H) resin and the solution concentrated to a syrup. This on paper chromatography gave strong spots corresponding to guluronic as well as mannuronic acid (solvent H). The two respective lactones were detected on papers eluted in solvent G, the spot corresponding to guluronolactone being considerably stronger than the analogous spot on the chromatogram from the sulphuric acid hydrolysis of the weed.


GRAPH NO 5
FRACTIONATION OF ALGINIC ACID
ON DEAE CELLULOSE
mg / fraction
160
140
120
100
80
60
40
20
10
20
30
40
50
90
FRACTION NO
0.1 M
KCl
stepwise
1.0 M

Expt.4. Attempted Fractionation of Alginic Acid on DEAE-Cellulose.

A sample of alginic acid extracted from Laminaria digitata by Dr. D. Drummond and stored at room temperature for ca.1·5 year was used in this experiment.

Alginic acid (1·1 g.) was dissolved in water (60 ml.) containing the approximately equivalent quantity (0·36 g.) of potassium hydroxide. The rather cloudy, slightly alkaline solution was filtered and the filtrate allowed to drain into the DEAE-cellulose column (in the chloride form). The column was left for 2 hours before the elution was started. Stepwise elution with potassium chloride solutions of increasing concentration was then carried out as follows:

0·10 M-KCl 500 ml., 0·25 M-KCl 500 ml.,
0·50 M-KCl 750 ml., and 1·0 M-KCl 750 ml.

The eluate was collected in 28 ml. fractions and the polysaccharide content determined by the phenol-sulphuric acid method (44). A calibration curve for alginic acid was made up in advance from a portion of the same sample. As shown on Graph No.5 the material was eluted in two distinct fractions. The contents of the tubes corresponding to the respective peaks were combined and dialysed to remove potassium chloride. After concentration the material was isolated by freeze-drying. The yields were:

Fraction 1 0·19 g. (83% of the calculated content from the phenol-sulphuric acid method)

Fraction 2 0·89 g. (100% of the calculated content).

A portion (0·1 g.) of each of the two fractions was hydrolysed with 75% formic acid (15 ml.), using the same conditions as in Expt.3(2). Paper chromatography (solvent H) clearly showed that the hydrolysates of both fractions contained mannuronic and guluronic acid and their respective lactones in the same relative proportions (visual examination). It was intended to measure the viscosity of the two fractions. However both fractions of potassium alginate proved to be so insoluble that it was impossible to carry out these determinations.

Expt.5. Extraction and Isolation of Alginic Acid from Laminaria digitata (120).

The seaweed was collected at North Berwick in November 1961. The fresh wet weed (2 Kg.) was cut into pieces (ca. 1 x 1") and stirred at 60° for 30 minutes with water containing calcium hydroxide (20 g.). The greenish liquid was decanted off and discarded, and the residual weed washed several times with water until the latter was neutral. 0·2N-Sulphuric acid (2·5 l.) was added, the mixture stirred for 10-15 minutes and then the liquid discarded. Another portion of sulphuric acid of the same strength (2·5 l.) was added and the mixture left overnight at 0°. The acid was decanted off and the weed washed with water and squeezed by hand until the

washings were neutral. 3% Sodium carbonate solution (5 l.) was added to the residual weed and the mixture digested at 40-60° with occasional stirring during 3 hours. The resulting brown-green jelly was diluted with water (20 l.) and stirred overnight at room temperature. Portions of 2-4 l. were withdrawn, diluted with an equal volume of water and stirred vigorously until a homogeneous mixture was obtained. Centrifugation and subsequent filtration by suction gave a clear light yellow solution. This solution of sodium alginate was poured into excess ca.N-hydrochloric acid with vigorous stirring. The precipitated gelatinous alginic acid was isolated in two ways. Some was washed exhaustively with water until neutral and free from chloride ions (2-3 days). This was then freeze-dried to a fluffy white solid. The remainder of the acid was washed with aqueous ethanol of increasing alcoholic concentrations (ca. 15-30-50%) until free from chloride. The washing was then continued with water-ethanol-ether mixtures of gradually decreasing aqueous content. Finally the alginic acid was triturated with alcohol-ether and with ether alone. The product was dried overnight on filter paper and then over phosphorus pentoxide in a vacuum desiccator. This product was slightly cream coloured and more lumpy than the freeze-dried material. Final yield of the dried material, 52·3 g. (Percentage yield can not be given since wet weed was used as starting material).
$[\alpha]_D$-137° (c, 0·40) (freeze-dried); $[\alpha]_D$-139° (c, 1·16)

(ether-dried). The optical rotations were measured in 0·1N-sodium hydroxide solution. The neutralisation equivalent for the freeze-dried and ether-dried samples were 199 and 198 respectively, estimated by titration with 0·05N-sodium hydroxide to phenolphthalein end point.

Expt.6. Preparation of Alginic Acid Dipropionate (121).

Alginic acid (20 g., dried for 2 weeks over phosphorus pentoxide in vacuo) was added in small portions to formamide (150 g.). Stirring of the suspension for 1 hour at 40-50° resulted in the formation of a thick jelly-like paste. Pyridine (200 g.) was added in small portions during 30 minutes at the same temperature under vigorous stirring and the mixture allowed to cool to about 30°. Propionic anhydride (200 g.) was then added under continuous stirring over 4 hours. Stirring was continued for a further 4-5 hours and the mixture finally left overnight at room temperature. The thick brownish solution was poured into 5 l. of N-hydrochloric acid, containing ca.500 g. of chopped ice. The ester precipitated as a cream coloured flocculent material that subsequently was filtered off, washed first with 0·1N-hydrochloric acid and finally with water. The pasty product was dried in vacuo over solid sodium hydroxide and then over phosphorus pentoxide to a cream-coloured crumbly solid. Yield, 26 g. (80%). For re-esterification the product (26 g.) was dispersed in pyridine (300 g.) to which was added propionic anhydride (50 g.).

The reaction mixture was allowed to stand at room temperature for one week under occasional shaking. The alginic acid dipropionate was precipitated by pouring the viscous solution into excess light petroleum (b.p. 60-80°) (ca. 1.5 l.). After filtration, washing with light petroleum and drying the ester was obtained as an off-white powder-like material 28 g. (86%). $[\alpha]_D$-166° (c, 0.50, in pyridine). Repeated re-esterification gave no further increase in the weight and no change in the specific rotation. The product was nearly completely soluble in diglyme.

Expt.7. Diborane Reduction of Alginic Acid Dipropionate (122).

Alginic acid dipropionate (12 g.) partly dissolved in dry diethylene glycol dimethylether (diglyme) (200 ml.) was mixed with sodium borohydride (20 g.) in diglyme (100 ml.). Boron trifluoride etherate (100 g.) in diglyme (250 ml.) was added in 25-30 ml. portions from a separating funnel over a period of 2-2.5 hour. After each addition of boron trifluoride etherate solution the separating funnel was replaced by a rubber stopper and the flask shaken by hand until the next addition. When all the boron trifluoride etherate had been added, the flask was left stoppered for 2 days at room temperature under occasional shaking. Ice was then added (vigorous effervescence) and water (300-400 ml.) followed by dilute sodium hydroxide to neutral reaction. The mixture was concentrated at 40°/1 mm. pressure to ca.150 ml. N-Sodium hydroxide

solution (150 ml.) was added to make the final concentration 0·5N with respect to sodium hydroxide; this gave a final pH of about 10. The mixture was kept at 60-70° for 2 hours. A small amount of insoluble material was removed by filtration and the filtrate dialysed and concentrated. Methanol was added several times with subsequent evaporations to remove last traces of borate. Finally the mixture was concentrated to ca.100 ml. and added to a stirred mixture of 25% ether in methanol (900 ml.). The precipitate after filtration (G-4 glass filter) and drying weighed 4·9 g. (75%). $[\alpha]_D$-87·5° (c, 0.40 in 0·1N-sodium hydroxide). For estimation of the neutralisation equivalent a part of the reduced material was converted into the acid form by treatment with IR-120(H) resin and isolated by freeze-drying. Aliquots dissolved in carbon dioxide-free water were titrated with 0·05N-sodium hydroxide (phenolphthalein end point). An equivalent weight of 2000 was obtained. This corresponds to an uronic anhydride content of 176 x 100/2000 = 8·8%. Uronic anhydride estimation by decarboxylation resulted in a content of 9·6% uronic anhydride. Paper chromatography of the reduced material after acid hydrolysis (N-sulphuric acid for 6 hr. at 100°) revealed spots corresponding to mannose and gulose (solvent B) and also a few weak slow moving acid spots, probably aldobiouronic acids. In addition three fast moving weak spots were detected with $R_{Mannose}$ values 1.65, 2·00 and 2·50 respectively, in solvent A. A trace spot with the colour and

mobility of xylose was also detected.

The three fast-moving weak spots previously revealed, had R_F, 0·56 (brown), 0·66 (brown) and 0·84 (pink), (solvent A, aniline oxalate spray).

The reduced alginic proved to contain n-propoxyl (5·2%), (kindly determined by Mr. S. S. H. Zaidi, employing the specific method of infrared spectroscopy (147) clearly differentiating between methoxyl, ethoxyl, iso- and n-propoxyl etc.).

Expt.8. Attempted Enzymic Hydrolysis of the Reduced Alginic Acid with Hemicellulase (146).

The reduced alginic acid (0·1 g.) was dissolved almost completely in water (20 ml.). Commercial hemicellulase (10 mg.) was added, the mixture shaken well and transferred to a dialysis tube with some drops of toluene. The dialysis tube was kept in distilled water which was stirred continuously and changed at intervals. After 3 days at 30° the dialysates were combined (ca. 2 l.) and concentrated. Paper chromatographic examination of the resulting syrup in solvents A and B revealed weak spots travelling at the speed of, and with the colour of (aniline oxalate spray) mannobiose and xylose as well as some slow-moving oligosaccharides. However, no spot with the mobility of mannose or gulose was detected. The enzyme itself on paper chromatography did not give rise to any spot. The residual material in the dialysis tube was

hydrolysed and examined chromatographically (solvent E). Spots of mannose and gulose were revealed and in addition a trace spot with the colour and mobility of authentic xylose.

A sample of the original alginic acid and of the seaweed Laminaria digitata were hydrolysed (N-sulphuric acid for 8 hours at 100°) and examined chromatographically in solvent A and B. The hydrolysate of alginic acid did not give rise to any pentose spot, while the hydrolysate of the weed gave a trace spot corresponding to xylose.

Expt.9. Partial Acid Hydrolysis Experiments.

(a) Portions of the reduced polysaccharide (10 mg.) were heated with sulphuric acid (1 ml.) of varying strength (0·5N, 0·25N, 0·1N and 0·05N) at 100° or 80° for varying time intervals (1, 2 and 5 hours). In all cases paper chromatographic examination of the hydrolysates revealed the presence of mannose and gulose, the latter being present in relatively larger quantities in the hydrolysates resulting from the weaker acid treatments (0·1N and 0·05N). In no case was the yield of oligosaccharides very satisfactory. Replacement of sulphuric acid with oxalic acid appeared to result in a slightly better yield of oligosaccharides.

(b) In a larger scale experiment reduced alginic acid (3 g.) was hydrolysed with 0·1N oxalic acid (300 ml.) at 90° for 2 hours. Some brown undissolved material was filtered off and dried, (0·15 g. = material A). The filtrate after concentration

at 35° to ca.75 ml. was treated with ethanol (6 volumes) and the mixture left overnight. Precipitated polysaccharide was filtered off and dried (0·98 g., = material B). The supernatant solution was concentrated to ca.100 ml. and neutralised with calcium carbonate. Subsequent treatment of the filtrate with IR-120 resin and evaporation afforded a syrup (1·80 g. after drying over phosphorus pentoxide).

Samples of materials (A) and (B) were subjected to hydrolysis and paper chromatography in solvent E. Material (A) proved to contain little or no carbohydrate (trace of mannose) whilst the hydrolysate of material B revealed a strong spot corresponding to mannose and a very weak spot of gulose. Paper chromatography of the syrup obtained from the partial hydrolysis showed the presence of approximately equal quantities of mannose and gulose (solvent E). A fair amount of low mobility higher oligosaccharides was also detected in addition to two distinct oligosaccharide spots, the one moving at the rate of mannobiose in solvent A and B, and the other (the stronger spot) with $R_{Mannobiose}$ 0·79 and 0·82 respectively in the two solvents. The three fast-moving spots (Expt.7) were also revealed (faintly).

Expt.10. Isolation and Investigation of the Major Oligosaccharide from the Partial Hydrolysate.

The syrup (1·80 g.), obtained in the preceding experiment, was subjected to separation on 3MM paper (ca.twenty sheets,

23 x 45 cm.) in solvent B (3 days elution time). The major oligosaccharide (X) was extracted from the filter paper with water after being located by spraying control strips. It proved not completely pure and was re-chromatographed in the same solvent. Oligosaccharide (X) was then obtained as a chromatographically and ionophoretically pure white solid (60 mg., M_G, 0·62 in borate buffer, pH 10). It was, however, contaminated with some water- and alcohol-soluble non-carbohydrate material from the filter paper and was not obtained in a crystalline condition. Work is in progress to remove the contaminant and it is hoped to crystallise the oligosaccharide. A hydrolysate on paper chromatography (solvent B) revealed spots corresponding to mannose and gulose, the latter being weaker than the former. However, on development of the chromatogram with silver nitrate and ethanolic sodium hydroxide (141) or with periodic acid-benzidine (145), a new faster spot ($R_{Mannose,Gulose}$ 1·80 (Solvent A)), slowly appeared. Pure gulose, when heated with acid under the conditions used in hydrolysing the oligosaccharide revealed on paper chromatographic examination the same fast moving spot, in all probability 1,6-anhydrogulose (142), in addition to the main gulose spot. One spot only, corresponding to gulose, was revealed with aniline oxalate spray.

The degree of polymerisation (D.P.) of the oligosaccharide (X) was estimated according to Timell's modification (143) of the method of Peat, Whelan and Roberts (144), employing phenol-

sulphuric acid (44) instead of the anthrone reagent. The oligosaccharide (X) (3.4 mg.) was dissolved in water (25.0 ml.). To 1·00 ml. of this solution was added 1% aqueous sodium borohydride (1·00 ml.). To a second 1·00 ml. of the same solution was added 1% sodium borohydride in N-sulphuric acid (1.00 ml.), "inactive borohydride." The two solutions were allowed to stand for 1 hour at room temperature. 4% Aqueous phenol (1·00 ml.) followed by concentrated sulphuric acid (5·00 ml.) were then added to each solution. After 0·5 hour at room temperature the optical density was read at 490 mμ against a blank prepared in the same way as the test solutions, but with water replacing the oligosaccharide solution. A number of tests were run and the average optical density of the colour formed by the reduced and the non-reduced oligosaccharide was 0·374 and 0·705, respectively. The D.P. is defined as $Q/Q-1$ where Q is the ratio of the optical densities given by the non-reduced and the reduced solution. Thus in the present instance Q = 0·705/0·374 =1·89, and accordingly the D.P. = 2·1.

To estimate the sequence of the two sugars in oligosaccharide (X), it (ca.3 mg.) was dissolved in water (0·5 ml.) and left overnight at room temperature with sodium borohydride and boric acid (ca.3 mg. of each). The solution was neutralised with 1R-120 resin, filtered, concentrated and freed from borate by additions of methanol and subsequent evaporation. The residue was then subjected to hydrolysis and paper chromatography

in solvent E. This revealed the presence of mannose as the only reducing sugar. When the chromatogram was developed with reagent b, in addition to mannose, the presence of L-gulitol (=D-sorbitol) was revealed and clearly distinguished from mannitol, both alcohols being run as control.

DISCUSSION.

Epimerisation experiments on uronic acids (Expt.1). When the presence of L-guluronic acid in alginic acid was recorded in 1955 (107) this was the first time that a rare uronic acid had been encountered as a component sugar in a polysaccharide. And since this acid is the C_5 epimer of D-mannuronic acid, the possibility of epimerisation at carbon 5 under the alkaline conditions used for the extraction of alginic acid should be considered. This is especially important in view of the recent investigations of Fischer and Schmidt (123). These workers found that simple uronic acids in aqueous solution at pH 7, in contrast to aldonic acids, easily underwent epimerisation at the carbon atom adjacent to the carboxyl group. They in fact say (123): "Schon das Eintrocknen von Tropfen ihrer neutralen Lösungen auf Papierchromatogrammen durch heisse Luft genügt zur teilweisen Epimerisierung." In alkaline solution the C_5 epimerisation reaction is accompanied by the well known C_2 epimerisation (Lobry de Bruyn - Alberda van Ekenstein rearrangement). In the present experiments mannuronic and glucuronic acids as sodium salts were subjected to the conditions claimed by these latter workers (123) to cause epimerisation at carbon 5. Under these conditions no spots corresponding to the C^5 epimers of these two acids could be detected by paper chromatography. But when the lactones of the two acids were heated under alkaline conditions similar to those employed

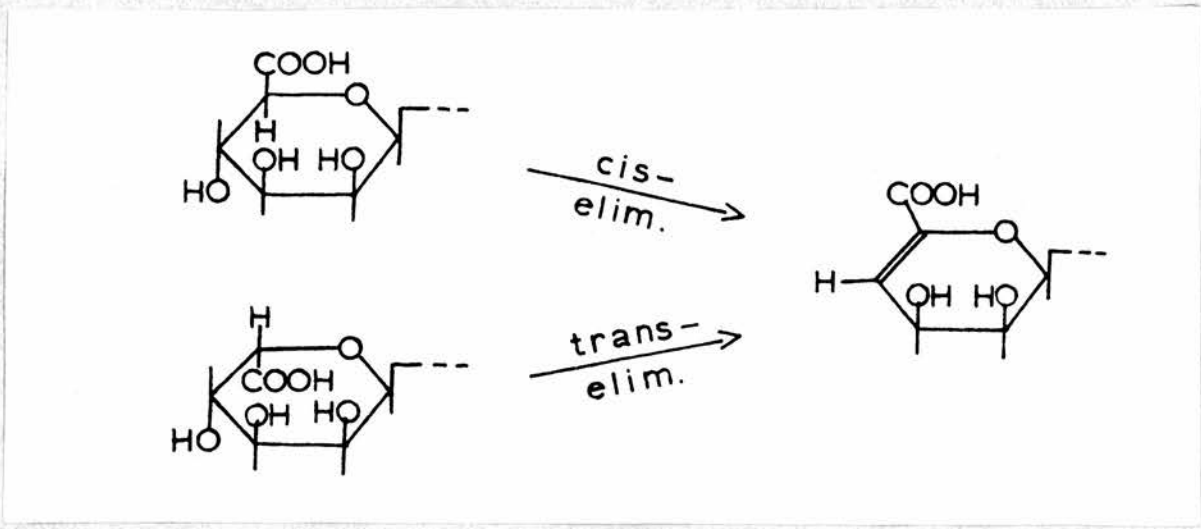
in the extraction of alginic acid (Expt.2) a significant C_5 epimerisation was found to take place (paper chromatography). This was not the case when the methyl ester methyl glycoside of mannuronic acid was subjected to the same treatment. This derivative of mannuronic acid with its substituted reducing group more closely resembles the units in alginic acid than does unsubstituted mannuronolactone, since the reducing groups of the individual residues in the polyuronide are substituted by linkage with adjacent units. It seems unlikely from this evidence that epimerisation at C_5 at the polysaccharide level is caused by the alkali used during the extraction. Proof that guluronic acid units are indeed present in the native alginic acid was obtained by acid hydrolysis of the weed itself (Expt.3). The resulting syrup was shown by paper chromatography to contain a considerable proportion of guluronic in addition to mannuronic acid.

Alginic acid is not the only polysaccharide containing two C_5 epimeric uronic acids. Hoffmann and coworkers (124) in 1956 reported the presence of L-iduronic acid in addition to D-glucuronic acid in chondroitin sulphate B. It is now thought, however, that these two acids do not occur together in the same polysaccharide molecule, and Jeanloz (125) succeeded in conclusively characterising the uronic acid component of pure chondroitin sulphate B as L-iduronic acid, the glucuronic acid found by earlier workers being due to contaminating polysaccharides. Evidence has been produced

(126) that the chondroitin sulphate A and B are stereoisomers differing only in containing C_5 uronic acid epimers. Identical unsaturated aldobiouronic acids (with double bond between C_4 and C_5 in the uronic acid moiety) were obtained from the two chondroitin sulphates by treatment with Flavobacterium enzymes.

Neukom and Deuel (127) found in a commercial pectinase preparation an enzyme which attacked pectin (but not pectic acid) and gave rise to the formation of oligouronic acids containing a 4,5-unsaturated uronic acid residue in the non-reducing moiety. It has been shown recently (128) that the same unsaturated uronic acid unit can be obtained by the action of sodium methoxide on the dimethyl ester of methyl 4-α-D-galacturonosyl-D-galacturonic acid.

Alginic acid on treatment with certain enzymes also appears to give rise to the same type of unsaturated oligosaccharides (129)(130). However, as far as the writer is aware these enzymic studies have not been applied to the structural investigations of this polysaccharide, and furthermore only crude or partially purified enzyme preparations were employed. These workers illustrate their results with D-mannuronic acid and ignore the implications of the presence of L-guluronic acid in alginic acid. It should be pointed out that a pure enzyme in view of its probable stereospecificity should distinguish between a cis and a trans-elimination reaction. Thus, conversion of a mannuronic acid unit to a



4,5-unsaturated uronic acid involves a cis-elimination of H and OH, while in the case of a guluronic acid residue a trans-elimination takes place. Consequently if alginic acid is a mixture of individual chains consisting solely of mannuronic acid residues or guluronic acid residues respectively, a pure enzyme should degrade the one type of chain leaving the other intact. In contrast if the two C_5 epimeric acids occur in the same molecule the enzyme should selectively attack the one type of epimer and thus degrade the whole molecule.

Attempted fractionation (Expt.4). Alginic acid has previously been fractionated by McDowell (131) who obtained alginate preparations of different viscosities by fractional precipitation with manganous chloride followed by calcium chloride. These fractions were not investigated from the chemical point of view. Haug (132) succeeded in separating alginic acid into a guluronic acid-rich and a mannuronic acid-rich fraction by addition of aqueous potassium chloride to a sodium alginate

solution. Polymer material containing mainly mannuronic acid was preferentially precipitated while the polysaccharide remaining in solution contained relatively more guluronic acid than the starting material. Repetition of this procedure (110) failed to yield polymeric material containing only mannuronic or guluronic acid. In order to try a new procedure, fractionation of alginic acid was attempted on a column of DEAE-cellulose by elution with potassium chloride solutions of increasing concentrations. A separation was indeed obtained since polysaccharide was eluted in two distinct peaks (Graph No. 5, p.107). However, the material from each peak on hydrolysis and paper chromatographic analysis proved to contain mannuronic as well as guluronic acid in approximately the same relative proportions. It is possible that this was merely a molecular weight fractionation. However, the solubility of both fractions in water and in alkali was so small that their viscosities could not be determined. Potassium alginate is generally regarded as a water-soluble substance, but in this instance freeze-drying yielded completely insoluble material. The fact that no chemical fractionation was obtained on DEAE-cellulose is obviously no criterion of the homogeneity of the sample of alginic acid.

Vincent (138) subjected alginic acid to partial hydrolysis and separated from the hydrolysates oligo-uronic acids which contained both guluronic and mannuronic acid

residues. However he offered no evidence, apart from chromatography, that the separated fragments were indeed single oligo-uronic acids and not mixtures of acids with the same chromatographic mobilities. The extreme difficulty of separating pure oligo-uronic acids, coupled with the high acid stability of alginic acid, makes the acceptance of this work as unequivocal proof that this polysaccharide contains both uronic acids in a single molecule difficult. Indeed Vincent's analyses of the ratio of the two acids present in his separated oligo-uronic acids serves to illustrate the experimental difficulties encountered in this work. Jayme and Kringstad (139) reported the isolation of a crystalline di-mannuronic acid by continuous electrophoresis of a partial acid hydrolysate of alginic acid. These workers, apparently unaware of the presence of guluronic acid in alginic acid, did not present further proof that their oligo-uronic acid in fact contained mannuronic acid only.

In the present studies it was hoped to obtain further evidence by conversion of alginic acid into a neutral polysaccharide. This would be considerably more amenable to partial acid hydrolysis and furthermore the derived neutral oligosaccharides should be more easily separated as pure individuals by standard chromatographic techniques. With this end in view alginic acid was extracted from freshly collected Laminaria digitata and converted into the 2,3-di-O-propionate prior to reduction with diborane.

The seaweed was collected in November and although the content of alginic acid at this time of the year is at its minimum, L.digitata is so rich in this polyuronide that a satisfactory yield was obtained. The weed was extracted immediately after collection by the method of Black et al. (120) with the exception that the product was isolated as the free acid instead of as the calcium salt (Expt.5). Difficulty was experienced in manipulating the large volumes of gelatinous alginic acid and it was found quicker to isolate it by washing with ethanol-water, ethanol and drying with ether than to wash it with water until free from chloride ions and then freeze-dry the polymer, although the product obtained by the latter route had a more delicate appearance. The isolated material was further dried for several weeks in vacuo over phosphorus pentoxide at 0°, since alginic acid when stored at room temperature tends to depolymerise (133). The neutralisation equivalent obtained (198 to 199) by titration with standard alkali appears somewhat high and indicates the presence of ca.1 mole of water for every uronic anhydride unit. This is difficult to explain in view of the very intensive drying of the polysaccharide before the titrations were carried out.

Alginic acid in its normal state is chemically rather inert and seems to require "activation" before acylation can be carried out. Percival (134) achieved this by swelling the alginic acid for 15 minutes in strong mineral acid (concentrated hydrochloric or 50% sulphuric acid). The acid was removed by

washing with water which in turn was replaced by glacial acetic acid. The thus "activated" alginic acid was acetylated (33·0% acetyl, theory 33·1%) with acetic anhydride in the presence of sulphuric acid (0·3%) as catalyst. Schweiger (135) acetylated alginic acid by a similar procedure using perchloric acid as the catalyst. In the present experiment (Expt.6) the alginic acid was brought into a swollen reactive form by treatment with formamide (121). The swollen alginate was suspended in pyridine and the acylation then effected with propionic anhydride. The product was re-propionated until constant rotation was attained. The dipropionate of alginic acid rather than the diacetate was prepared since Smith (122) reports that alginic acid diacetate is not sufficiently soluble in the ether-type solvents generally used in diborane reductions.

Diborane is a powerful reducing agent for organic compounds such as aldehydes, ketones, carboxylic acids and nitrites. Esters are said to react very slowly under the conditions (room temperature) used for the reduction of carboxyl groups. The marked difference in the reductive properties of the borohydrides and diborane is attributed to the acid-base functions of these reducing agents. The alkali borohydrides are bases, and react through a nucleophilic attack of the borohydride ion on an electron deficient centre. Diborane, on the other hand, is a Lewis acid and preferentially attacks groups of high electron density (137). A large excess

of diborane (ca.10 times) was used in the present experiment (Expt.7) as well as a long reaction time (2 days). Under these conditions ca.90% of the carboxyl groups were reduced to primary alcohol groups. The neutralisation equivalent, (2000), indicated a residual uronic anhydride content of 8·8% in good agreement with the uronic anhydride content obtained by decarboxylation (9·6%). The apparent presence of xylose in a total hydrolysate of the reduced material, also reported by Smith (122), is difficult to explain, since the starting material gave no indication of any pentose sugar. Other workers (136) however have reported xylose to be present in hydrolysates of alginic acid. But it should be emphasised that chromatographic evidence only has been presented for the presence of this sugar. If a minor degree of decarboxylation takes place during the drastic conditions necessary for the hydrolysis of alginic acid lyxose, not xylose, would be produced from mannuronic as well as from guluronic acid. The contaminant in the acid as well as in the enzymic hydrolysate of the reduced polysaccharide gave a pink spot (aniline oxalate) with the mobility and colour of authentic xylose and a different mobility from authentic lyxose. In the absence of more rigid proof its identity as xylose rather than a degradation product can only be tentatively assumed.

The other three fast-moving spots with Rf values 0·56, 0·66 and 0·84, respectively, are difficult to account for. The fastest moving compound, showing up pink with aniline oxalate,

was not revealed when spraying with alkaline triphenyl tetrazoliumchloride, indicating a substituted 2-position. Since it has been shown (140) that acetylated hydroxyl groups in certain steroid derivatives on diborane reduction have been converted to the corresponding ethoxy groups ($R \cdot O \cdot CO \cdot CH_3 \longrightarrow R \cdot O \cdot CH_2CH_3$) it was thought that the same reaction also might occur to a minor extent during the conditions used for the diborane reduction of an acylated polysaccharide. If so, a proportion of n-propoxygroups should be formed from the propionyl residues in the present case. Such residues would be acid stable and it is assumed that a mono- and in particular a di-propyl monosaccharide would have a high mobility on a paper chromatogram. Analysis of the reduced alginic acid gave a propoxy value of 5.2% corresponding to one propoxy group for every seventh sugar unit. While this is not significant in the present structural studies, it may be of importance in other reductions of carbohydrates by this method.

Action of commercial hemicellulase on the reduced alginic acid (146) resulted in the formation of a very small proportion of oligosaccharides, the one occurring in the largest amount travelling at the same rate as mannobiose on a paper chromatogram. Partial acid hydrolysis proved more satisfactory for the production of oligosaccharides, although this procedure also led to a relatively small yield of these sugars. The general picture of a paper chromatogram after a rather mild acid hydrolysis (e.g. 0.25N sulphuric acid for 1 hour at 100°)

was a high proportion of monosaccharides, (mannose, gulose and trace quantities of the fast-moving propoxy-derivatives), a high proportion of material hardly moving from the starting line of the chromatogram and a comparatively small proportion of lower oligosaccharides. When more dilute acid (0·1N or 0·05N) was used, the gulose spot became stronger relative to the mannose spot. It was originally assumed that reduced alginic acid would more or less resemble a β-1,4-linked mannan, in its general properties, but this was not the case. A β-1,4-linked mannan is generally a water-insoluble and acid-resistant polysaccharide (148)(149), whereas the reduced alginic acid was water-soluble and rather labile even to weak acid. This fact might possibly be taken as support that at least a part of the alginic acid is not composed of separate chains of β-1,4-linked mannuronic acid and of guluronic acid respectively, in view of the acid sensitivity of some of the mannose linkages in the reduced polysaccharide.

Oxalic acid was finally chosen for the large scale partial hydrolysis, as this acid seemed to produce a slightly better yield of oligosaccharides. After hydrolysis with 0·1N oxalic acid at 90° for 2 hours, polymeric material, ca. a third of the original weight, was precipitated with ethanol. This material appeared on acid hydrolysis to consist almost entirely of mannose together with a very small proportion of gulose. But in view of the ready conversion of gulose to its 1,6-anhydride under acid hydrolytic conditions (see below) the

proportion of gulose is probably higher than would appear at first sight.

The low molecular material was obtained as a syrup after neutralisation and concentration of the aqueous alcoholic supernatant. This was separated by chromatography on thick paper and the major oligosaccharide (X) after re-chromatography, was obtained ionophoretically and chromatographically pure. It was not obtained crystalline and was contaminated with some non-carbohydrate material from the filter paper, which made the recording of the optical rotation impossible. It is unfortunate that lack of time has not permitted purification of this oligosaccharide before presentation of the thesis.

A hydrolysate of oligosaccharide (X) on paper chromatography showed the presence of mannose and in addition a weaker spot of gulose. This could therefore correspond to a trisaccharide comprising two moles of mannose and one of gulose. However, the chromatographic and ionophoretic mobilities of oligosaccharide (X) were rather high for a trisaccharide. When the chromatogram of the hydrolysate was developed with silver nitrate, however, a new fast-moving spot was revealed. Richtmyer et al. (142) report that gulose on treatment with acid is partially converted to the 1,6-anhydrosugar. And in fact after subjection of chromatographically pure gulose to conditions identical to those employed in the hydrolysis of oligosaccharide (X), paper chromatographic examination

revealed the fast-moving spot detected in the hydrolysate of the oligosaccharide after development with silver nitrate-ethanolic sodium hydroxide or with periodate-benzidine, but not with aniline oxalate. From this evidence it is highly probable that the fast-moving spot on the chromatogram of the oligosaccharide hydrolysate is derived from gulose and that oligosaccharide (X) contains equal amounts of mannose and gulose.

The estimation of the degree of polymerisation (D.P.) was based upon the principle that the phenol-sulphuric acid reagent reacts with a reducing sugar with the formation of a colour, but not with a sugar alcohol. The relationship between absorbance and concentration is linear within a certain range (44)(143) and consequently by reading the optical density obtained by the action of the phenol-sulphuric acid reagent on the oligosaccharide before and after borohydride reduction, the D.P. can be found. It should be remembered that borohydride reduces the reducing moiety in a disaccharide to the corresponding alcohol. Thus for a disaccharide the absorbance before reduction should in theory be twice as high as after reduction. However, the different hexoses do not invariably give rise to exactly the same colour intensity when subjected to the phenol-sulphuric acid test, and therefore with mixed oligosaccharides the D.P. may be slightly more or less than a whole number. In view of this the D.P. of 2.1 obtained for oligosaccharide (X) is clear evidence that it is

indeed a disaccharide.

The sequence of the two sugars in the oligosaccharide (X) was obtained by borohydride reduction and subsequent hydrolysis and paper chromatography. One reducing sugar only, corresponding to mannose, was detected, together with an alcohol moving at the speed of L-gulitol(D-sorbitol) but differently from mannitol. Evidence has been obtained in the foregoing experiments that oligosaccharide (X) is a mannosylgulose. Methylation studies and periodate oxidation of this disaccharide should reveal whether it is 1,4-linked as would be expected from previous investigations of alginic acid.

Apart from the work of Vincent (138) this is the first time that direct evidence has been presented for the existence of mannuronic and guluronic acid linked together in the same molecule of alginic acid. However, this mannuronic-guluronic acid link may well exist only in a proportion of the closely related polysaccharides which appear to constitute alginic acid. That this polyuronide is not a single homogeneous polymer has been clearly demonstrated by fractionation experiments, where fractions rich in the one or the other of the two acids have been obtained. Some interesting findings have recently been reported by Frei and Preston (150) who have obtained evidence for the existence of two major components of alginic acid. These workers succeeded in extracting a considerable amount of a polymannuronic acid material with hot water and obtained after subsequent treatment with sodium

chlorite a polymer apparently rich in guluronic acid. They suggest that the polyguluronic acid occurs mainly in the cell wall as a sort of hemicellulosic material while the mannuronic acid polymer is located in the intercellular mucilage of the brown algae. By employing the technique of X-ray diffraction Frei and Preston found that the X-ray diagram previously obtained for alginic acid (108) and hitherto assumed to be the diagram of poly-D-mannuronic acid is in fact that of poly-L-guluronic acid. According to these authors this is due to the fact that in a mixture of the two polymeric acids the crystallisation of the polyguluronic acid tends to suppress that of the polymannuronic acid. It may well be correct that the cell wall contains mainly guluronic acid-rich alginic acid and that the intercellular alginic acid mainly constitutes polymannuronic acid. However, the author feels it as an over-simplification when Frei and Preston claim that nearly pure polymannuronic acid can be extracted with water and that the polyguluronic acid in the cell wall can be obtained subsequently by chlorite treatment. It appears more likely that alginic acid consists of a family of polymers containing varying proportions of the two acids.

CONCLUSION.

The present investigations have confirmed earlier evidence that the guluronic acid units of alginic acid do not arise from C_5-epimerisation during the alkaline conditions used for extraction of this acid. Fractionation of alginic on DEAE-cellulose did not result in any chemical separation although two distinct fractions were obtained, (possibly of varying molecular weight). Alginic acid extracted from fresh *Laminaria digitata* after conversion into the di-n-propionate was subsequently reduced with diborane. The derived nearly neutral polysaccharide was subjected to partial acid hydrolysis. From this hydrolysate a pure disaccharide was separated. This was shown to be a mannosylgulose but was not fully characterised. The presence of this disaccharide as the major oligosaccharide in the partial hydrolysate demonstrates that a proportion of mannuronic-guluronic acid linkages indeed exists in alginic acid. While these results throw additional light on the complex structure of the alginic acid molecules, considerably more information should be obtained from methylation and periodate oxidation studies on the neutral polysaccharide and its degradation product isolated from the partial hydrolysate.

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